ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Fiscal Year 1981

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
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ANNUAL REPORT

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ANNUAL REPORT OF PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

October 1, 1980 Through September 30, 1981

The Director has maintained his busy schedule leading the Institute into new outlooks and activities. In addition to the Council and Committee meetings there was added the full responsibility for the Recombinant DNA Guidelines and the Advisory Committee. Much time went into investigation and decision on violation of the Guidelines. There was also added responsibility for a Performance Review Board for Senior SES members of NIH.

In February, NIAID sponsored a meeting with representatives of constituent professional societies and voluntary associations to review programs and budgeting outlooks. This was well attended and appreciated. Dr. Whitescarver did an excellent job of organizing the meeting and the informational material made available to the attendees. Also in February and March, the Director attended the meetings of the Joint Council on Allergy, the Association of Medical School Microbiology Chairmen and the Council of the American Society for Microbiology to hear their views of NIAID's activities and brief them on the budgetary outlook for FY 1982.

Moral support and advice was given to the Asthma and Allergy Foundation in its efforts to better represent victims of these problems by organizing a meeting of Foundation officials with Directors of several of the Asthma and Allergic Diseases Centers. Productive discussions on outreached programs and how these could best be implemented were useful to all participants including NIAID staff.

A highlight of the year was the General Staff Meeting in March at which EEO awards were made to several staff members. These are new awards.

The Director's busy schedule as a leader in the field of research on allergies, immunology, microbiology and infectious diseases is reflected in his many speaking engagements. A list of these follows:

> Malaria; The King of Diseases. October 9, 1980. Practitioner's Society Meeting, New York, New York.
>
> Recombinant DNA - Risk Assessment Speech, October 9-10, 1980. Bethesda, Maryland.
>
> Dedication Remarks of the New Immunology Laboratory Building. October 22, 1980. Scripps Clinic and Research Foundation, La Jolla, California.
>
> Introductory Remarks, A Community Reaching Out, Can We Improve the Ends as well as the Means? November 19, 1980. Center Director, Asthma & Allergy Foundation, Bethesda, Maryland.
>
> Liaison Between the NIAID and the Professional Societies and the Voluntary Health Agencies. February 12-13, 1981. Council of Allergy and Immunology, Hyatt-Regency Hotel, Washington, D.C.

Opening Statement, Association of Medical School Microbiology Chairmen, February 21-22, 1981, Tampa, Florida.

Opening Remarks to receive C. William O'Neill Distinguished Service Award from the Marietta Area Chamber of Commerce, March 9, 1981. Marietta, Ohio.

Seminar Remarks, Malaria; The King of Diseases, March 24, 1981. Department of Microbiology at the University of Alabama in Birmingham, Alabama.

Introductory Remarks: The Kinyoun Lecture. "Murine SLE A Model for Autoimmunity" by Dr. Frank J. Dixon. April 3, 1981. Wilson Hall, NIH, Bethesda, Maryland.

"Frontiers in Medicine" The Restless Tide: Persistent Challenge of the Microbial World. April 20, 1981. The Smithsonian Institution, Washington, D.C.

Malaria; The King of Diseases, May 13, 1981. To give the first Stephen I. Morse Memorial Lecture at Downstate Medical Center, Brooklyn, New York.

The Restless Tide: A Persistent Challenge of the Microbial World. May 15, 1981. Harvard University, Department of Tropical Public Health, Boston, Massachusetts.

Streptococcal Cell Walls: The Middle Years, June 2, 1981. The Rockefeller University, Dr. Maclyn McCarty's retiring affair. New York, New York.

Philadelphia, the founding Fathers, and SLE. June 7-9, 1981. Franklin Plaza Hotel, Philadelphia, Pennsylvania.

Role of Public Policy in Preventive Medicine. June 15-16, 1981. Presented at a symposium on Preventive Medicine in the Coming Decade Medical Advisory Council Meeting, Merck Sharp & Dohme International Amsterdam, Netherlands.

Remarks for The Board of Directors Annual Meeting of the Asthma & Allergy Foundation of America, New England Chapter. June 24, 1981. Weston Golf Club, Weston, Massachusetts.

Remarks for Secretary Schweiker. June 25, 1981. Stone House, NIH.

Would the Wassermann Reaction Survive Peer Review: Conference on Bacterial Virulence and Pathogenesis. July 27-29, 1981. RML, Hamilton, Montana.

Remarks for the Asthma & Allergy Foundation. Rural Letter Carriers August 6, 1981. Baltimore, Maryland.

The year end marked the ending of the 16-year career of the Deputy Director, Dr. John R. Seal, in the NIAID. Dr. Seal had many accomplishments during these years. In the past year one of the significant accomplishments was proposing the presentation of the Hepatitis B Story to the NIH Director's Advisory Committee and organization of the program put on by the Director, Dr. Krause, and by Drs. Robert Purcell of LID, NIAID, and Harvey Alter of the Clinical Center Blood Bank. The written text has had wide dissemination and will be further used in describing the contributions of basic research to disease prevention. Dr. Seal had planned to retire but has agreed to defer this to help organize an overall NIH program on prevention related research.

A major personnel happening was the move of Mr. Kenneth Cooke, Assistant Executive Officer, to the National Library of Medicine. Dr. Sheldon Cohen gave the invitational von Pirquet Lecture at Georgetown School of Medicine in May. Dr. Wallace Rowe received the General Motors Cancer Research Award ($100,000) in June. Ms. Rose Lieberman received the highest civilian award in the Department, the Distinguished Service Award in July, and Dr. Anthony Fauci was recognized by the Secretary for his receipt of the Arthur S. Flemming Award. The whole Institute was saddened by the death of Dr. Robert J. Byrne after a long illness. He had filled several important roles and at the time of his death was scheduled to become the Director, Extramural Activities Program.

Special Assistant to the Director

The extent and effectiveness of interaction with constituent groups was increased as has been noted above. The publication, NIAID Profile 1981, was compiled and distributed. The Special Assistant played an invaluable role on a part-time temporary assignment to the extramural program, taking major responsibility for Council activities and a reorganization that involved the Data Analysis Branch. He has also been extraordinarily effective in liaison with Congressional staff, innovating opportunities for these to become more familiar with NIH. One such innovation was the arrangement for several staffers to attend a Study Section meeting.

Research Manpower Development Staff

This is a new part of the Office of the Director. It was created to succeed the Training Branch of the Extramural Activities Program and to broaden the responsibilities to emcompass all training conducted by the Institute. Dr. William Bennett joined the Institute in August to head this new activity. His first activity was to arrange for the Fellowship Review Committee to meet in September.

Office of Program Planning and Evaluation

During Fiscal Year 1981, this office continued to serve the Director as the advisor on the development, analysis, and evaluation of the Institute's programs; provided support and liaison to the program managers on coordinating, integrating and articulating long-range program goals and strategies; and served as the principal advisor on legislative and administrative developments which included projecting their impact on NIAID programs and operations. The major documents for which the OPPE had lead responsibility were the Institute's Annual Research and Evaluation Plans and associated reports. It also responded to requests for trans-NIH committees and other sources for program information and assisted in preparing material required for the Congressional budget presentations.

Following a management analysis of OPPE functions and extensive internal discussions on how the OPPE could best meet the increasing demands placed on it, a decision to reorganize the office was made late in the year. When fully implemented, the OPPE will consist of four sections; Planning, Evaluation, Classification, and Data Control. The Classification and Data Control Sections will be staffed by personnel from and perform similar functions as the Program Analysis and Data Control Sections which were previously part of the Program Analysis and Evaluation Branch, Extramural Activities Program. The remaining two sections will be staffed by a combination of transfers of staff from within the Institute and recruitment actions. Dr. Marilyn Bach's assignment with OPPE, as part of the IPA program, expired and she is completing the year working in the Immunology, Allergic and Immunologic Diseases Program. Dr. Alfred Webb's appointment as a reemployed annuitant will expire at the end of FY 1981 thereby ending his long and productive association with the OPPE.

Considerable staff effort was expended in planning and managing an evaluation project in conjunction with the Office of Specialized Research and Facilities, namely a Workshop for Chairpersons of Institutional Biosafety Committees. Key recommendations were that many experiments which were controlled, at that time, by the Guidelines be made exempt from the NIH Guidelines. The IBC chairpersons felt that the risks associated with these experiments are negligible, and that exemption of experiments would reduce unnecessary paperwork by approximately 90%. This recommendation was discussed by the Recombinant DNA Advisory Committee and an overwhelming majority favored taking formal action that would materially reduce or eliminate the paperwork and reporting functions; significant changes in the Guidelines were promulgated in June 1981. A second key recommendation was that a planned continuation and expansion of the evaluation study be broadened to include safety issues other than recombinant DNA. This recommendation emanates from the viewpoint of the majority of IBC chairpersons that there are many larger issues on the horizon of concern to institutions (e.g., chemical carcinogens and etiologic agents) which are apparently more hazardous than recombinant DNA. Accordingly, NIAID staff is collaborating with the Director, Division of Safety, NIH, in developing a revised approach to Phase II of the study.

Office of Specialized Research and Facilities

This office was disestablished during FY 1981 and the remaining functions transferred to the Office for Recombinant DNA Activities.

Office of Recombinant DNA Activities

The NIH Guidelines for Research Involving Recombinant DNA Molecules continued to evolve as major actions recommended by the Recombinant DNA Advisory Committee (RAC) were approved by the Director, NIH. The RAC met on January 8-9, April 23-24, and September 10-11, 1981. Major revisions of the NIH Guidelines were promulgated in the *Federal Register* on November 21, 1980 and July 1, 1981. Under the latest revisions, most experiments involving *E. coli* K-12, *Saccharomyces cerevisiae*, and *Bacillus subtilis* host-vector systems are exempt from the Guidelines. P1 physical containment continues to be recommended for these experiments. The July 1, 1981 revisions also include an Appendix G, which specifies containment conditions for the cloning in E. coli K-12 of genes coding for the biosynthesis of toxins for vertebrates. Levels of physical and biological containment are specified based on the LD_{50} of the toxin. Prior to the recent revision, the cloning of most toxin genes was prohibited by the Guidelines.

The Guidelines specify that the Director, NIH, is responsible for authorizing under procedures specified by the RAC, large-scale experiments (i.e., those involving more than 10 liters of culture) for recombinant DNAs that are rigorously characterized and free of harmful sequences. During FY 81, eight proposals to exceed the ten liter limit were recommended for approval by the RAC at its January and April, 1981 meetings, and approved by NIH, followed by a notice in the Federal Register. These proposals included two submissions from the academic community, and six, which were handled on a confidential basis, from the private sector. The latter, all voluntary submissions from Genentech, Inc., Hoffmann-La Roche, Burns-Biotec Laboratories, Schering-Plough Corporation, and Cetus Corporation, involve attempts at large-scale production of human leukocyte and fibroblast interferons, and bovine growth hormone.

The Director, NIH, accepted a recommendation of the RAC to establish a Large-Scale Review Working Group. The Working Group is to advise the RAC on procedures and facilities design pertaining to large-scale operations, and on the performance of local Institutional Biosafety Committees in reviewing physical containment facilities. The Working Group is to assit the RAC in development of general recommendations on large-scale containment, and not for purposes of regulatory actions directed at individual companies. This Working Group held an organizational meeting on April 23, 1981, to discuss approaches to its assignment, and met again on September 9, 1981.

A Working Group on Revision of the Guidelines was established as a result of recommendations of the RAC at its April 1981 meeting. This Working Group is charged with reviewing (a) the present need for the Guidelines in their existing form and procedures, as opposed to a voluntary standard of practice; (b) continued applicability to recombinant DNA technology; (c) currently recommended levels of containment; and (d) current processes and procedures impeding or facilitating research and/or industrial applications. The Working Group is to report back to the RAC its findings, conclusions, and recommendations for RAC review and consideration. Solicitation of public input and comment on the Working Group's recommendations beyond publication in the Federal Register is planned. This Working Group met on June 1 and July 9, 1981, and developed recommendations for major revisions of the Guidelines, as well as a draft document on its assessment of the risks associated with recombinant DNA research. The recommendations of this Working Group were considered by the full RAC at its September 1981 meeting. Final action on a major revision of the Guidelines is expected during the next fiscal year.

Responsibility for the NIH Risk Assessment Plan was transferred from the Office of Specialized Research and Facilities to the Office of Recombinant DNA Activities (ORDA) during FY 81. The proposed first annual update was published for comment in the Federal Register near the beginning of the fiscal year. The comments were taken into consideration, and the first annual update was published in the Federal Register on June 10, 1981. The updated plan reports on the current status of tests on prokaryotic host-vector systems performed in situations simulating accidental spills in the laboratory, in a model sewage treatment system, in mice and cultures simulating the mouse gastrointestinal system, in germfree mice, and in humans.

ORDA continued to interact with other Federal agencies regarding recombinant DNA research and associated activities. The Director, ORDA, serves on the USDA Recombinant DNA Committee, which met on a frequent basis during FY 81. This Committee assisted the NIH in the review of a proposal for the field testing of corn plants modified by recombinant DNA techniques. This proposal was approved by the NIH in July 1981. The Director, ORDA, served as a lecturer and discussion leader at a seminar on Science, Technology and Public Policy sponsored by the U.S. Office of Personnel Management at its Western Executive Seminar Center in Denver, Colorado, on August 5, 1981.

Issues of the Recombinant DNA Technical Bulletin were published in November and December 1980, and in April, July and September 1981. In April 1981, ORDA published Volume 6 of Recombinant DNA Research, which documents activities in regard to the NIH Guidelines during the period from January 1980 to December 1980. Detailed minutes of RAC meetings were prepared and distributed using ORDA's extensive mailing keys.

ORDA continued to interact with European poliy makers during FY 81. On January 14-15, 1981, the Director, ORDA, participated, as the United States representative, in the sixth meeting of the European Science Foundation Liaison Committee on Recombinant DNA Research. The role of this Committee is to foster a common set of standards for the conduct of recombinant DNA research among the countries of Europe and North America. The Director, ORDA, participated in the first meeting of the United States - Japan Cooperative Program for Recombinant DNA Research on February 4-5, 1981. At the meeting, there was an exchange of information on current national guidelines, risk-assessment, and development of new host-vector systems. The Director, ORDA, was a member of a U.S. delegation which attended a meeting on May 14-15, 1981, on Safety Aspects of Research on Recombinant DNA sponsored by the Economic Community (EEC). This meeting explored whether the EEC should issue a directive mandating the regulation of recombinant DNA research in the member countries. At the invitation of the government of Japan, the Director, ORDA, participated in a meeting of the Special Committee for Revision of (Japanese) Recombinant DNA Guidelines in Tokyo on August 25-27, 1981. Dr. Gartland appeared before the Committee and presented information on risk-assessment, the current NIH Guidelines and possible future developments.

ORDA presented information on the NIH Guidelines at a national conference on Recombinant DNA and the Federal Government on October 9-10, 1980, at a meeting of chairmen of Institutional Biosafety Committees on November 24-25, 1980, at a special seminar of the Biology Department of Brooklyn College on December 15, 1980, at the annual meeting of the American Society for Microbiology on March 4, 1981, at the 1981 International Conference on Genetic Engineering sponsored by the Battelle Memorial Institute on April 7, and at the American Industrial Hygiene Conference on May 28, 1981. Locally, the Director, ORDA, led a discussion on genetic engineering at the Paint Branch Forum in Prince Georges County on February 22, 1981.

Within the NIH community, ORDA provided orientation talks on the NIH Guidelines and NIH policies to the DRG Microbial Chemistry Study Section on November 5, 1980, to NIGMS staff on March 9, 1981 to staff of the Division of Safety on March 17, 1981, and to NIAID staff on May 21, 1981. In addition, the Director, ORDA, briefed the NIH Director's Advisory Committee on protection of proprietary information on June 8, 1981, and participated in a briefing of the Surgeon General designate on July 13, 1981.

Mrs. Betty Butler, who had served as lead secretary in ORDA since its establishment in 1976, left ORDA to become secretary to the Deputy Director, NIAID. Ms. Becky Connors has assumed these responsibilities in ORDA.

On June 22, 1981, the Director, NIH, delegated all authorities under the NIH Guidelines to the Director, NIAID.

Office of Research Reporting and Public Response

The ORRPR employs various approaches to accomplish its chief function -- dissemination of information about allergies and infectious diseases as well as NIAID's programs. These include production of publications, audiovisual materials, and exhibits; publicity about the Institute's scientific programs; and answers to public and Congressional inquiries by letter and telephone. In addition, the ORRPR provides extensive editorial and speechwriting support for Dr. Krause. To perform these tasks, the ORRPR realigned its staff this year to handle its responsibilities more efficiently while utilizing the skills and strengths of each individual.

Personnel -- Joan Hartman has joined the staff as a writer-editor assigned particularly to stimulate press activity and to answer inquiries from the news media. Margot Hardesty has been hired to manage the "library." Under contract, Reynard Beimiller continues to advise the staff on the layout and design of nonroutine publications. Tom Coleman, expert in medical communications, completed his 2-year appointment in July.

Publications -- A new fact sheet on "Reye's Syndrome" was produced. Revisions of the pamphlet "Allergies: Questions and Answers" and of the fact sheets "Mononucleosis," "Tuberculosis," "Schistosomiasis," and "Systemic Fungal Disease" were also published. A fact sheet on "The Common Cold" has been revised and is ready for publication. The covers for ORRPR's series of allergy pamphlets for patients have been redesigned, and revisions of the texts are under way. "Insect Allergy," "Drug Allergy," and "Poison Ivy Allergy" are ready for printing; "Food Allergy" and "Dust Allergy" are in the process of being revised.

The ORRPR staff assisted the editing scientific monographs on "New Initiatives in Immunology," New Initiatives in Immunology: Summary and Recommendations," and "Sexually Transmitted Diseases: 1980 Status Report of the NIAID Study Group," as well as the profile book "NIAID 1980." In addition, 35 NIH Record stories, 5 articles for NIH News and Features, and 6 "Search for Health" columns were written.

Approximately 300,000 NIAID publications were distributed this past year. This number includes special mailings, those furnished to health fairs, scientific meetings, and the Consumer Information Service, and requests by telephone and mail. It also includes copies of "Allergies: Questions and

Answers," which recently were placed in display cases at selected supermarkets throughout the United States. The 20 locations were chosen for their proximity to NIAID Asthma and Allergy Disease Centers.

Public and Congressional Inquiries -- The ORRPR composed approximately 600 individualized letters in answer to public inquiries, responded to over 4,000 telephone inquiries from the public, and answered 60 Congressional inquiries. Records show that 110 letters were sent in response to requests in accordance with the Freedom of Information Act.

Cngressional Reports -- NIAID sections for six special reports to the Congress were prepared on genetic diseases, diabetes, digestive diseases, cystic fibrosis, arthritis, and sexually transmitted diseases.

Scientific Meetings and Information -- Exhibits were produced and manned by ORRPR staff for the following meetings: the American Public Health Association, Detroit; the American Society of Tropical Medicine, Atlanta; the American Academy of Allergy, San Francisco; and the American College of Allergy, the Association of Military Surgeons, and the Housing and Urban Development Health Fair, all in Washington, D.C. Most of these exhibits had either a videotaped message or a slide show in addition to a display of appropriate NIAID publications. Posters and teaching slides based on the report "New Initiatives in Immunology" were also prepared for use at exhibits and meetings.

New lighted displays have been placed in the seventh floor elevator lobby in Building 31. One section, with space for three transparencies, holds information dealing with research by grantees; the other section is used for current publications and news items relevant to the Institute.

The ORRPR staff prepared the publicity for the Kinyoun Lectures, established 2 years ago by Dr. Krause to emphasize the interdependence of infection and immunity. This year's lecturers were Dr. Leroy Hood, California Institute of Technology, and Dr. Frank J. Dixon, Scripps Clinic and Research Foundation. The ORRPR staff also developed publicity for other newsworthy activities associated with NIAID this past year. Among these were the "telephone hook-up" press conference from the American Academy of Allergy meeting in San Francisco to Chicago and Bethesda and the press and TV coverage of the Sloane prize for basic research awarded to NIAID's Dr. Wallace Rowe.

Liaison with AAFA -- This office has established a closer working relationship with the editorial staff of the Asthma and Allergy Foundation of America. A special column in AAFA's quarterly newsletter "In Touch" features information from NIAID writted by ORRPR staff. Further cooperative efforts involve distribution of selected publications from both sources. For example, the AAFA is helping to distribute NIAID's book "Asthma and Allergies: An Optimistic Future" and the pamphlet "Allergies: Questions and Answers." The ORRPR is distributing AAFA's pamphlets "Allergy in Children," "Skin Allergy," and "Handbook for the Asthmatic."

Film Production -- A 28-minute feature film on genital herpes has been completed, and plans are being made for its distribution first to television stations and later to interested organizations for group showings.

Library -- ORRPR's library continues to provide the NIAID staff with current scientific information published in the fields of allergy and infectious diseases. Currently, 102 journals and 7 newsletters are received. In addition, 40 textbooks were ordered this year and 20 more were donated. A popular innovation by Mrs. Hardesty has been the production (usually twice a week) of an in-house "clip sheet" containing newspaper and magazine items relevant to the Institute's work.

1981

Annual Report
Immunology, Allergic and Immunologic Diseases Program
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REPORT OF THE DIRECTOR

IMMUNOLOGY, ALLERGIC AND IMMUNOLOGIC DISEASES PROGRAM

Fiscal Year 1981

A. Administrative Summary

1. Organization and Functions

The Immunology, Allergic and Immunologic Diseases Program (IAIDP) was initiated on October 1, 1976, coincident with the reorganization of NIAID into major programs characterized by special professional functions. With this change the IAIDP assumed the responsibility for research, training, and conference activities that formerly were assigned to the Allergy and Immunology Branch of the Extramural Programs and contract activities in transplantation immunology and immunologic research resources previously administered within the Collaborative Research Program.

During FY 1981, the IAID Program operated according to the following organizational plan:

Office of the Director (OD)

Director-Sheldon G. Cohen, M.D.
Deputy Director-Bernard W. Janicki, Ph.D.
Special Assistant to the Director-Dorothy D. Sogn, M.D.
Assistant for Program Management-George R. Jenkins

Clinical Applications Section (CAS)
Head-Daniel I. Mullally, M.D., M.P.H.

Allergy and Clinical Immunology Branch (ACIB)

Chief-Robert A. Goldstein, M.D., Ph.D.
Special Assistant-Thomas T. Hubscher, Ph.D.
Program Associate-Joel D. Rosenthal

Immunology and Immunochemistry Branch (IIB)

Chief-Bernard W. Janicki, Ph.D.
Program Officer-John G. Ray, Jr., Ph.D.

Genetics and Transplantation Biology Branch (GTBB)

Chief-Henry Krakauer, M.D., Ph.D.
Program Associate-Justina S. Grauman, M.D.
Serum Bank Manager-Anne Heath

Each of the three IAIDP Branches assumes responsibility for administering initiated research grants and collaborative research contract awards, developing programmatic initiatives and new projects, serving on trans-NIH coordinating committees and taking active roles in the development and conduct of workshops

workshops and conferences in areas of the biomedical and biological disciplines and the clinical medical specialties with which they are concerned.

All three Branches are also involved with programs related to Research Career Development Awards and training, i.e., individual and institutional Fellowships and the Young Investigator Research Grant Awards designed to assist and encourage investigators in early stages of their careers to develop research interests and capabilities in immunology. In addition, the Allergy and Clinical Immunology Branch administers Allergic Disease Academic Awards which are designed to further the careers of mid-level investigators on career tracks in academic and research allergy, and this year initiated the Clinical Investigator Award. The purpose of this new program is to provide opportunities for young individuals trained as clinicians with demonstrable interest and aptitude in investigation to develop the requsite basic research backgrounds and skills in the fields of immunology and allergy, and in turn to increase the pool of clinical investigators applying knowledge and relevant basic biomedical sciences to clinical problems in allergic and immunologic diseases.

The IAID Program also assists investigators by procuring and providing certain reference standard and research reagents not otherwise available from commercial sources. These have included human and mouse histocompatibility typing sera, purified allergens, and antisera to lymphocyte subsets. Program staff's responsibility and awareness of needs to assist constituent fields are reflected in the expansion and development of several new endeavors in this area.

2. Centers and Program Projects

The IAIDP/NIAID Clinical Research Centers Programs are now comprised of 22 Centers. Eighteen Asthma and Allergic Disease Centers (AADC) are located at the Scripps Clinic and Research Foundation, Mayo Clinic, NIAID NIH Clinical Center, and university medical school and hospital centers at California/San Francisco, Colorado, Northwestern, Kansas, Tulane, Johns Hopkins, Harvard/Brigham and Women's Hospital, Michigan, Creighton, New York (NYU), SUNY Buffalo, SUNY Stony Brook, Duke, Texas/Dallas, Wisconsin and the most recent addition at Tufts. The 4 Centers for Interdisciplinary Research on Immunologic Diseases (CIRID) at the university medical school and hospital centers at California/LosAngeles (UCLA), Georgetown, Washington (St. Louis) and Rochester have undergone an initial special three-year program review in accordance with the congressional directive responsible for the establishment of this program. In addition to the conduct of basic biomedical research and clinical investigations in asthma, allergic and immunologic diseases and the application of basic data to attacking clinical problems in diagnosis, treatment and management of patients with these disorders through multi-disciplinary collaborative approaches, these Centers have been focusing efforts on the development of educational and community activities, outreach regional programs for both professional groups and the laity.

Through the provisions of the Lymphocyte Biology Program the IIB has set the goal of attaining comprehensive knowledge of the life history of immunocompetent cells and of the genetic and phenotypic factors that determine cellular fate and function in vivo and in vitro. Program project studies (Lymphocyte Biology Centers) in this defined area are in progress at five universities located at Rockefeller, Yeshiva, Texas/Southwestern (Dallas), Harvard and Washington (St. Louis).

To encourage investigations of the underlying mechanisms of immunologic disease and to enhance basic knowledge relevant to the etiology, prevention and management of such disorders effective from one of two disciplinary approaches, clinical immunology or immunopathology, the ACIB has established a special program, Mechanisms of Immunologic Diseases. Program projects are currently in effect at university medical school and hospital centers at Harvard (Children's Hospital), Johns Hopkins, Scripps Clinic and Research Foundation and Northwestern.

By annual announcements (RFA) new institutions are invited to compete for available funds with ongoing Centers at the time of submission of renewal applications. An additional feature of this Centers - Program Project endeavor has been inter-Branch facilitation and support of investigator exchanges between institutions comprising this network. Rapid developments and progress in research on physiologic function, chemistry and the genetics of the immunocompetent cells have not only been rewarding in revealing fundamental knowledge but additionally have pointed to relevant leads for effecting clinical relevance. Accordingly, staff exchanges are designed to favor applications of basic biomedical science data to clinical investigations.

3. Special Research Grant Announcements

During FY 1981 IAIDP issued the following Requests For Research Grant Applications (RFA):

Asthma and Allergic Disease Centers (discussed above).

Projects in Mechanisms of Immunologic Disease (discussed above).

Program Projects in Lymphocyte Biology (discussed above).

Mechanisms in Food Allergy. The objective of the ACIB in these studies is to effect research directed at the elucidation of basic mechanisms essential for an understanding of the role of food antigens in causing a variety of allergic and/or adverse reactions. By addressing basic immunologic and/or other pathophysiologic mechanisms of causation it is anticipated that this important but underserved clinical area can be accomplished.

Transplantation Immunology. The objective of the GTBB in these studies is to take advantage of the momentous advances in technical procedures and in the understanding of molecular cellular processes to bypass remaining hazards associated with immunosuppressive therapy and rejection of organ transplants. Focus should be given to the application of the most up-to-date concepts and techniques of immunology to the evaluation of the immune system of recipients in all circumstances attendant to transplantation. Of especial concern will be clarification of immunoregulatory balance and modulation of immunologic activity.

As a result of the issuance of a GTTB initiated RFA on the genetic and structural characterization of minor non-MHC and tissue alloantigens, a study has been initiated at the Wistar Institute in Philadelphia. This project was designed to stimulate research on histocompatibility determinants not in the MHC (i.e. minor histocompatibility loci), and not expressed on lymphocytes. This is a difficult and underserved area of potentially great significance to transplantation and should compensate for the deficiencies of histocompatibility determinations previously focused entirely on products of the MHC and only antigens expressed on lymphocytes. The limited predictive power of matching donors and recipients at the MHC loci may be due in some cases to disturbances of immunoregulation induced by immunosuppresive regimens resulting in autoimmunity or vigorous reaction to normally weak antigens, or to the presence of polymorphic antigens coded by loci other than MHC or those specific for the transplanted tissue.

Other ACBI sponsored joint Institute Announcements have resulted in funding of research initiatives with NIAMDD in dermatology and diabetes, NEI in ocular immunology and NHLBI in hypersensitivity lung diseases.

4. Collaborative Projects

Through the facilities of the American Type Culture Collection the IIB has been engaged in the establishment and conduct of an hybridoma cell bank involving certain cell lines produced by the fusion of spontaneously proliferating and differentiating cells, e.g., myeloma and antibody-producing lymphocytes. Procedures for their systematic acquisition, characterization, storage, and distribution of designated types are progressing. Specific monoclonal antibody-producing hybridoma cell lines are made available to the concerned research communities and to contractors charged with providing the GTBB serum bank with typing reagents. In this latter aspect an important and noteworthy advance has been effected in the development of hybridoma production of monoclonal tissue specific antibodies. Forty-three hybridoma cell lines have thus far been donated and of these six have already completed processing procedures and are available for distribution. It is anticipated that 20 to 40 cell lines per year will be acquired and processed for a total bank inventory of 500 at the completion of this initial five-year period.

The ACIB has expanded its resource capabilities by effecting the special preparation and acquisition of new reference standard materials including: a battery of fungal antigens and human antisera relating to the identification of etiologic factors of hypersensitivity pneumonitis, selected lyophilized preparations of food antigens for standardized challenge testing in clinical studies on food allergy, a complement (C3) standard for biologic assays, and in collaboration with WHO an antihuman IgG-horseradish peroxidase agent for use in the enzyme linked immunosorbent assay (ELISA). To support this ACIB effort by quality control a contract has been awarded to the Mayo Foundation for the establishment of an Allergen Resource Reference Laboratory.

By contract support the GTBB has been assisting in the work of the International Bone Marrow Transplantation Registry maintained at the Mt. Sinai Medical Center in Milwaukee. Data collection is designed to provide pertinent information on immune processes and immunologic problems associated with bone marrow transplantation and thus represents a unique resource to the medical science and clinical communities coming under the GTBB's purview.

The clinical trial of skin testing with major and minor penicillin derivatives in hospitalized adults is now entering the second of two years. Reagents are available for testing at each of 8 partcipating university medical school and hospital centers located at California/Los Angeles (UCLA), Washington (Seattle), Northwestern, Colorado, Rochester, Cornell, Walter Reed Army Medical Center and the NIAID NIH Clinical Center. At issue is whether skin testing with major and minor determinants of penicillin can predict hypersensitivity reactions to administration of penicillin (or its analogs) in history positive and in history negative hospitalized adults. If this predictive value is shown the study may further demonstrate whether a positive or negative history adds information in this respect, beyond that gleaned from skin tests. Of the required 500 history positive patients in those thus far enrolled, preliminary data show that approximately 85% of such patients are skin test negative and may recieve penicillin without danger of immediate life threatening allergic reactions. Of the required 3500 history negative patients, preliminary data show that approximately 2% are skin test positive leading to the assumption that such patients are at high risk for immediate life threatening allergic reactions to penicillin.

Renewal support was given for continuation of the trans-NIH contract project on tabulation and analysis of immunoglobulin sequence data; to publish sequence data into a single reference resource, to develop further concepts concerning immunoglobulin synthesis and diversity, and to expand the data base to include nucleic acid sequences of immunoglobulin chains and the inclusion of sequence data on HLA, H-2, Ia, and related molecules.

A collaborative study at the Sidney Farber Cancer Institute is concerned with investigation on immunodepressant effects of a series of conventional drugs including corticosteroids, cytotoxic agents, antilymphocyte/thymocyte serum or globulin and total lymphoid irradiation. Indications have been obtained that some of these agents do selectively depress subsets of T lymphocytes. This can serve as the first step in the elucidation of the mechanisms of action of these conventional immunodepressant drugs and agents. In addition these data provide the insights that will be needed for the proper employment of subsets specific monoclonal antibodies as immunomodulating agents. In conjunction with certain pilot studies on the use of these agents being conducted presently within a very short time it may be possible to conduct a full scale clinical trial of these monoclonal antibodies in transplantation and in a variety of autoimmune disorders.

An advance in a contract supported project has resulted from the work of a study group on organ specific alloantigens directed to review evidence that tissue specific antigens, independant of the MHC antigens, have demonstrable actions in graft rejections. Findings and opinions on technologies available for characterization of tissue antigens generated evaluatory and developmental concepts applied to the ongoing IAIDP hybridoma studies at the American Type Culture Collection.

During 1981 the following Requests For Proposals (RFP) were issued in order to initiate new projects in identified areas of need and to invite competition for awards for ongoing work at the time of submission of renewal applicant proposals.

Preparation of alloantisera specific for H-2 murine cell membrane antigens: To Provide a Continuous Source of Well Characterized, Functionally Active, H-2 Alloantisera For Distribution To Qualified Investigators And For Quality Controlled Testing of Other Acquired Antisera; awarded to Mayo Foundation.

Development of methods to prepare bulk supplies of alloantisera specific for mouse cell surface determinants. To continue the development of methodology for the production of alloantisera specific mouse cell surface antigens, the preparation of relatively small quantities of such antisera, the storage of these reagents and their distribution to selected qualified investigators, and the provision of quality control testing of other acquired antisera; awarded to Sloan-Kettering Institute for Cancer Research.

Screening, Characterization, and Characterization of Histocompatibility Testing Antisera Capable of a More Effective Definition of Transplantation Antigens of the Ethnic Subpopulations in the United States: To overcome difficulties in tissue typing of non-caucasians, an outcome that is unsatisfactory from the theoretical immunogenetics standpoint and in terms of its practical consequences in organ and tissue transplantation; to be awarded.

Development and clinical evaluation of a rapid cellular cross matching technique suitable for use in cadaveric organ transplantation. To develop a cellular cross matching technique sufficiently rapid to be useful in estimating histocompatability between recipient and cadaveric donors in renal and other organ transplantation and to supplement incomplete information determined by serologic cross matching for free formed antibodies; to be awarded.

Allergen resource reference laboratory. For the purpose of acquiring and making available standardized materials, allergens and other immunopharmacologic reagents, for clinical investigations and to serve as a reference laboratory for the production, analysis, assay and quality control of a variety of immunologic-allergenic reagents and materials and as a centralized reference laboratory facility for immunodiagnostic tests; awarded to Mayo Foundation.

5. Research Resources

The IIB has provided the opportunity for qualified researchers with the best quality antisera of available cellular antigenic determinants for basic immunologic investigations of mouse systems: Ia, Ly, H-2, Thy-1,Qa-1 and Tl. Support of this programmatic interest is a development of the recognition of the role of murine strains as the focal point of research in experimental immunogentics because of the effect of this series of closely linked genes on tissue transplantation, susceptibility to viral infection and cell interactions during immune responses. Thirteen hundred ml. were distributed to 194 qualified investigators, 132 U.S. and 62 foreign.

A newly developed immunologic H-2 complex reagent can be supplied to grantees and qualified investigators studying manipulation of the immune mechanisms by antigen recognition control and immunotherapy through the work of the IIB. Newer specificities H-2 antisera related to tissue histocompatabiliy and specific Ia antisera involved in immune response mechanisms have been identified. Ly antisera specific for thymus dependent lymphocytes and for nonhistocompatability cell marker studies, tumor specific antibodies and theta cell antisera have been developed, banked and distributed. Eighty nine ml. alloantisera were distributed to 144 qualified investigators, 34 U.S. and 10 foreign.

A manual comprising various tissue typing techniques, methods for freezing tissue cells and the acquisition of antisera is available and has been provided to qualified researchers and medical libraries.

Experimental reagents have been evaluated and experimental methodologies in tissue antigen extraction, identification and applicability have been developed by contracts (see Collaborative Projects Section).

An author-journal index of literature concerned with hybridoma technology, basic research and catalog of hybridoma strains has been developed and maintained by the IIB.

6. Histocompatibility Serum Bank Activities

The inventory and ordering procedures for bank sera and the evaluation of reference laboratory reports is functioning on an automated system. The work of the Serum bank has involved 400 sera offers, 165 of which represented rare specificities, and 450 users.

Plans are underway for developing a data acquisition and analysis capability in the serum bank for the purpose of conducting inhouse studies on pooled national data in the area of immunogenetics.

The production of redesigned typing sera trays has been completed and their release effected. These comprise basic clinical trays and an extended tray to meet research needs. It is anticipated that this step may effectively serve to initiate transition of resources developed by NIAID to commercial suppliers. More recent approaches to DR serum typing indicative of promising advancements has resulted in the design and production of DR locus typing tray. Laboratories working in this newly developing area serve as sources of appropriate serum sample contributions.

Reevaluation of needs and usage of homozygous typing cells has resulted in redefinition of contracts for their acquisition. As a result of inhouse evaluatory studies the "NIH technique" for assay has been developed in an attempt to standardize usage and to recommend an accurate scoring method to achieve greater reproducibility and comparability among laboratories.

7. Immunology Study Group

The work of the Immunology Study Group organized during FY 1980 has been completed. Comprised of 98 members working in 6 panels dealing with regulatory mechanisms, hybridoma technology, cell cloning, molecular genetic approaches to immunologic systems, cell membrane antigens and effector mechanisms was co-chaired by Drs. Baruj Benacerraf of Harvard and William Paul of NIAID LI with Dr. Dorothy Sogn of this Program serving as Executive Secretary. Focus was given to the state of immunologic knowledge in those areas where important developments were considered most likely to occur and identified areas where special support is needed. Priorities among these determinations were then assigned. Prior to publication, the panel reports and recommendations were considered by a Subcommittee of the Institute Advisory Council (NAAIDC) in preparation for presentation to full Council. The full report entitled New Initiatives in Immunology and the shorter Council Summary and Recommendations for funding in each of the six important areas were published in January 1981. The first mentioned document has been given wide distribution to the immunology community.

8. Awards

Dr. Sheldon G. Cohen, IAIDP Director, received the Clemens von Pirquet Lectureship Award at Georgetown University on May 22 for contributions to allergy and clinical immunology, and the Association for the Care of Asthma M. Murray Peshkin Lectures Award on March 6, 1981.

Three medical scientists whose work has been supported by the NIAID received the 1980 Nobel Prize in medicine or physiology for studies in immunogenetics, Drs. Baruj Benacerraf of Harvard, George Snell of the Jackson Memorial Laboratory in Bar Harbor, and Jean Dausset of the College of France in Paris.

Dr. E. Donnall Thomas of the University of Washington and the Fred Hutchinson Cancer Research Center in Seattle whose work is supported by a NIAID Research Career Award recieved the General Motors Cancer Research Foundation Charles F. Kettering Award during June 1981 for pioneering work on bone marrow transplantation and its application to the treatment of acute leukemia and aplastic anemia.

9. Special Staff Projects

The Clinical Application Section has completed an epidemiologic study utilizing the medical record facilities at Children's Hospital National Medical Center of D. C. The observation presented by Dr. William Howard of that institution to the Section on Allergy of the American Academy of Pediatrics has been verified; the diagnosis of asthma indicated as a leading cause of admissions among children not under continuing outpatient care, with a tenfold increase noted over the past 25 years. Further study will be necessary to evaluate whether there may be demonstrable relationships to identifiable socioeconomic, environmental or etiologic and pathogenic factors to explain this phenomenon, and whether this may be only a local inner city phenomenon or represents a national trend.

The ACIB and CAS conducted a survey of medical schools in the U.S. and Canada in an approach to ascertain academic, clinical and teaching needs in allergy and clinical immunology. A 100% response of the 124 schools queried indicated deficiencies. In allergy 69 (56%) departments of internal medicine reported unsatisfactory situations, 59 without full-time staffs; 52 (42%) departments of pediatrics indicated unsatisfactory situations, 60 without full-time staff. In clinical immunology 54 (44%) departments of internal medicine indicated unsatisfactory situations, 38 without full-time staff; 60 (48%) department of pediatrics indicated unsatisfactory situations, 55 percent without full-time staff. Additionally, 14 departments of internal medicine and 18 departments of pediatrics were without part-time/salaried faculty in either allergy or clinical immunology.

The ACIB has been exploring possibilities with the Robert Wood Johnson Foundation for developing and implementing cooperative endeavors in outreach and community activities by NIAID Asthma, Allergic and Immunologic Disease Centers (AADC and CIRID).

The Kidney Transplant Histocompatibility Studies (KTHS) has entered the second phase of analysis which consists of indepth evaluation of factors that have been identified as significant in phase one. Included in these are the effect of race of the recipient, perioperative and preoperative blood transfusions, and of diabetes as a primary disease. Another very important component of the second phase of analysis is the development of a statistical model which may be used to predict the outcome of transplants in individual patients. With the use of this model it should be possible to select recipients and methods of treatment to optimize the probability of success. This statistical model is in the process of evaluation against the KTHS data base and also that of the Southeast Organ Procurement Foundation (SEOPF).

A formal collaboration has been established between the GTBB and the Medical Information Service of the HCFA End Stage Renal Disease Program to evaluate data principally on transplantation contained within the files of this federal agency. This task has been substantially completed acquiring identification of a population upon whom enough information could be made available to perform the requisite analysis and definition of the date of failure of the graft. Graft and patient survival were determined as a function of age, race, sex, and primary disease. In addition similar analysis of patient survival was performed for those patients on dialysis who had not yet been transplanted. The analysis have been submitted to the Endstage Renal Disease Program and plans for further cooperative endeavor are being developed.

10. Liaison and Outside Activities

IAIDP Staff have joined with the Asthma and Allergy Foundation of America (AAFA) and the Lupus Foundation of America (LFA) in developing and effecting joint endeavors for educational and community activities to further the causes served by these lay groups. Additionally, IAIDP staff members have been contributing to the work of professional and scientific societies and to the educational and service endeavors of medical schools and clinical institutions.

Dr. Robert Goldstein serves on the faculty of the George Washington School of Medicine and the medical staff of the G.W. University Hospital, Dr. Dorothy Sogn on the staffs of the Allergy Clinics at Walter Reed Army Medical Center and the NIH Occupational Health Unit, and Drs. Cohen, Goldstein and Sogn on the NIAID LCI Clinical Center staff.

Lectures to LFA Chapters were presented by Dr. Dorothy Sogn at Indiannapolis during October 1980 and at Boston during June 1981; Dr. Robert Goldstein in Philadelphia during December 1980; Dr. Sheldon Cohen in Wheeling during January 1981, and to the LFA annual National Council of Representatives meeting in San Francisco during July 1981 on the subject of Research in SLE.

Serving as visiting professors of medicine: Dr. Robert Goldstein at the Chester-Crozier Medical Center during January 1980, and Dr. Sheldon Cohen at West Virginia University-Ohio Valley Medical Center in Wheeling during January 1981.

Lectures to professional groups: Drs. Sheldon Cohen and Robert Goldstein at the Georgetown University CIRID Clemens von Pirquet Day program during May 1981; Dr. Sheldon Cohen to the Johns Hopkins Allergy Group during September 1980, the Brookdale Medical Center (Brooklyn) Pulmonary Diseases Course during October 1980, the Midwest Allergy Forum during October 1980, the Connecticut Thoracic Society during October 1980, the American Opthalmologic and Otolaryngologic Society of Allergy during May 1981, and the California Society of Allergy during September 1981.

Dr. Robert Goldstein served in committee chairmanship positions for the American College of Chest Physicians and the American Thoracic Society.

Dr. Cohen serves on the Board of Directors of the AAFA and the LFA, the Medical Advisory Council of the LFA, and with Dr. Dorothy Sogn on the AAFA Committee for Public Education.

Dr. Everett K. Spees, NIAID Consultant for the GTB group presented a paper on Preoperative but not Perioperative Blood Transfusions Improve Primary Cadaver and Living Related Renal Transplant Graft Survival, at the annual meeting of the American Society of Transplant Surgeons in Chicago, June 5, 1981. Justina Grauman lectured on the Analysis of KTHS Factors That Affect Graft Survival to the Public Health Service Officers Association in Boston on May 27, 1981. Dr. Henry Krakauer lectured on the Banking of Hybridomas at the American Type Culture Collection Conference in Rockville on June 7, 1981.

Under the direction of the ACIB, the annual AADC and CIRID Directors Workshop underwent a change in format at a joint meeting with key representatives and chapter presidents of the AAFA on November 19-20, 1981. Discussions were conducted on ways and means that Centers and AAFA Chapters might assume mutually supportive roles and joint activities in their respective geographic regions.

Through the work of the ACIB this Institute jointly sponsored a conference on Self-Management Programs for Asthmatic Children with the AAFA and the UCLA CIRID in Los Angeles during June 1981 and with the AAFA and the National Jewish Hospital and Research Center a postgraduate day on asthma and allergic disease for primary care physicians at the NIH Clinical Center during September 1981.

11. International Health Activities

Drs. Sheldon Cohen and Robert Goldstein serve on International Union of Immunological Societies (IUIS)-World Health Organization (WHO) Committees; Dr. Cohen the Committee on Standardization and Dr. Goldstein the Sub-Committee on Standardization of Allergens. Both attended meetings at WHO in Geneva during February 1981, and Dr. Goldstein the Paul Ehrlich Seminar in Frankfurt during September 1981.

Dr. Sheldon Cohen as a consultant to the WHO Immunology Unit visited in Geneva during January 1981, in Kuwait during April 1981, and participated in the International Union Against Tuberculosis-WHO meeting in Edinburgh during August 1981.

Drs. Sheldon Cohen, Robert Goldstein and Dorothy Sogn are developing the format for the WHO epidemiologic study on the prevalence and nature of asthma and allergic disease in developing countries, to be initiated in Kuwait, Venezuela and Thailand.

As consultant to the Ministry of Public Health in Kuwait, Dr. Cohen visited in that country during April 1981 to confer on plans for the development of an Allergy Center for diagnostic referrals, training and research to serve Kuwait and the Arabian Gulf area.

Dr. Goldstein served on the faculty of the WHO Immunology Course in Lausanne and lectured at the Universities of Montpellier, France and Bern, Switzerland during September 1981.

U.S.-Japan Cooperative Medical Science Program: The IAIDP accepted the charge from the U.S. and Japan delegations to expand the scope of this program to include immunology. As an initial step, Drs. Bernard Janicki and Sheldon Cohen of IAIDP with Dr. William Paul NIAID Intramural LI worked with Japanese counterparts to develop and convene a symposium on immunology in Tokyo on October 16-17, 1980. As a result of this interchange and related interactions among the U.S. and Japanese representatives an Immunology Board has been established. A long-term objective of this Board will be to study and elucidate cellular and molecular mechanisms of the immune response and to define effective approaches for manipulation of the immune system focusing on diagnosis, therapy and prevention of diseases due to disordered immune function. Tentative plans have been drawn up for the second symposium to be held at NIH during May 11-12, 1982.

U. S. People's Republic of China Program for Cooperation in the Science and Technology of Medicine and Public Health: IAIDP has been assisting the NIAID Director, Dr. Richard Krause, in his responsibilities as Chairman for the U.S. Section on Immunology within the Joint Health Committee of the Developing U.S.-P.P.R. Program. As a result of Dr. Krause's discussions with the P.P.R. Chinese counterpart Chairman, it was decided that specific studies for cooperative research would include HLA tissue typing and disease associations, allergy, immunity to infections, and specific problems in clinical Immunology. The first step in implementing this agreement was the visit of three Chinese allergists-immunologists to NIH during May 1981 and related visits arranged for them at the NIAID Asthma and Allergic Disease Centers at Johns Hopkins and Harvard Medical Schools and the Scripps Clinic and Research Foundations and to NIAID supported programs at Rockefeller and Cornell Universities. Discussions were held with the objective of defining possible areas where mutually supportive and cooperative investigations in allergy could be pursued.

12. Publications

The following publications are concerned with IAIDP related work, projects and programmatic activities.

Krakauer, H.: Applications of Monoclonal Antibodies Produced by Hybridomas to Diagnostic Immunology, Microbiology, 1981, in press.

Krakauer, H.: Central Banking of Hybridomas, In Vitro, in press.

Hartman, R., Krakauer, H., et al: Monoclonal Antibodies Specific for Human Polymorphic Cell Surface Antigens, I. Evaluation of Methodologies, Transplantation Proceedings, in press.

The continuing detailed analysis of the 5 year KTHS now in the second phase has resulted in the submission of two manuscripts for publication and a third in the process of preparation.

Spees, E. K. Jr., Grauman, J.S. and Krakauer, H.: The Effects Of Perioperative Blood Transfusions on The Outcome of Primary Living Donor Renal Transplants; An Analysis of The NIAID Kidney Transplant Histocompatibility Study, Transplantation (submitted).

Spees, E.K. Jr., Bailey, R.C., Ayres J., Summe, J.P., Grauman, J.S. and Krakauer, H.: The Effects of Perioperative Blood Transfusions on The Outcome of Primary Cadaver Renal Transplants; An Analysis of The NIAID Kidney Transplant Histocompatibility Study, Transplantation (submitted).

Spees, E.K. Jr., Grauman, J.D., and Krakauer, H. Bailey, R.C. and Summe, J.P.: Predictions of Outcome In Renal Transplantation by Use Of a Statistical Model, (manuscript in preparation).

A new GTBB catalog supplement for users of histocompatibility testing reagents that now includes homozygous typing cells has been distributed.

Proceedings of a GTBB conference has been published under the title of Organ Specific Alloantigens, edited by Lalezari, P., and Krakauer, H.: Transplantation Proceedings, 10:No. 3 (Supplement 1), 1980; also available in hardcover reprint, Grune and Stratton publishers.

Manual of Tissue Typing Techniques (revised) has been made available to qualified research immunologists and geneticists.

The ACIB has prepared and distributed a catalog of available reference standards and reagents for investigations in allergy and clinical immunology entitled NIAID Research Resources for Allergic and Immunologic Studies. It is planned to incorporate materials in this publication with that of comparable information available from the Food and Drug Administration and produce a NIAID-FDA combined resource catalog by collaborative effort.

The work of the Study Group on Immunology is represented in the following NIAID publications.

New Initiatives in Immunology, NIAID Study Group Report, 1981.

New Initiatives in Immunology, Summary and Council Recommendations, 1981.

Proceedings of the Joint NIAID-NHLBI Workshop on Basic and Clinical Aspects of Granulomatous Diseases have been published in book form; Boros, D. L., and Yoshida, R. editors, Elsevier North Holland, New York, 1980. Additionally, a summary version has been prepared for publication in American Review of Respiratory Diseases.

Based upon published material in the NIAID report of the Task Force on Asthma and the Other Allergic Diseases, the ACIB is working with the American Academy of Allergy Scientific and Educational Council in the development of manuscript material for a Primer in Allergy.

A cooperative effort is underway with NIAMDD in the development of a Handbook for the Lupus (SLE) Patient.

13. Conferences

During FY 1981 the following meetings, conferences and workshops relevant to program activities and functions were conducted by the IAIDP.

September 19, 1981	Workshop on Non-Ethnic White Antigens, NIH, Bethesda.
October 27, 1980	Workshop on Monoclonal Antibodies, Georgetown University, Washington.
November 19-20,1980	Asthma and Allergies; AADC and CIRID Directors and AAFA Joint Workshop, NIH, Bethesda.
February 25, 1981	Ad Hoc Advisory Group on Transfer Factor in the prevention of Varicella-Zoster Infection, Workshop, NIH, Bethesda.

March 21-24, 1981	Workshop on Non-MHC Antigens, American Association for Clinical Histocompatibility Testing, Orlando.
June 11-12, 1981	Self Management Educational Program for Childhood Asthma Workshop, UCLA CIRID and AAFA Workshop, Los Angeles.

Additionally IAIDP program support was given to the Following national and international conference and workshop endeavors.

November 1980	Midwest Autumn Immunology Conference, Minneapolis.
December 1980	Biological Significance of Immune Regulation, Seattle.
February 1981	Immunoglobuline Idiotypes and Their Expression, Salt Lake City.
April 1981	Problem Areas in the Vascularized Organs, Cambridge, England.
June 1981	Use, Availability and Patenting of Hybridomas and Genetically Engineered Organisms, ATTC, Rockville.
June 1981	International Conference on Lymphatic Tissues and Germinal Centers in Immune Reactions, Groningen, The Netherlands.
June 1981	Current Perspectives on the Immunology of of SLE Philadelphia.

Consensus Development Conferences: Two consensus development conferences have been scheduled and are currently in planning stages.

1. Relationship Between Defined Diet and Hyperactivity in Children, January 14-15, 1982. To be sought will be a definition of hyperactivity and determination as to whether hyperactivity can be affected by dietary manipulation.

2. Immunotherapy of insect sting hypersensitivity, May 6-7, 1982. At issue is the efficacy of hymenoptera venom and/or whole body extracts as antigenic agents for hyposensitization procedures in patients manifesting immediate hypersensitivity reactivity to stinging insects.

14. Budget

The following shows the distribution of support by award mechanism for the activities of the Program during FY 1981.

Immunology, Allergic and Immunologic Diseases Program
FY 1981 Awards

Award Mechanism	Number	Amount*
Research Grants	563	$ 61,207,000
Career and Career Development Award	48	1,804,000
Subtotal	611	63,011,000
Fellowship Awards	56	$ 835,000
Training Programs	31	2,272,000
Subtotal	87	$ 3,107,000
Contracts	38	$ 1,465,000
Total	736	$ 67,583,000

*Total costs, indirect costs estimated.

B. Scientific Summary

Programmatic interests of the Immunology, Allergic and Immunologic Diseases Program (IAIDP) are concerned with functions of the immune system in the maintenance of health and disordered immune function in disease. Within the spectrum of IAIDP activities are studies focusing on cellular and humoral antibody mediated aspects of immune phenomena, immunopathologic processes, hypersensitivity and allergic inflammation, immunogenetic relationships to disease and transplantation biology. Special attention is directed to original work and studies on structure, function and basic biology of the immune system and their applications to pathophysiologic alterations. Responsibility for involvement in these biomedical areas is accomplished by a variety of mechanisms that include investigator initiated research projects, research and development contracts, clinical investigative centers concerned with asthma, allergic and immunologic diseases, program projects dealing with lymphocyte biology and immunologic diseases, establishment of research resources, and distribution of reference standard reagent materials including the maintenance of a serum bank for histocompatibility testing materials.

To accomplish these objectives, the IAIDP functions in three organizational scientific branches;

1. Allergy and Clinical Immunology
2. Immunobiology and Immunochemistry
3. Genetics and Transplantation Biology

The work of these three IAIDP Branches is designed to favor program insights and alertness to important features of current status and new developments in basic and clinical immunology and allergy. Attempts are made to continuously monitor relevant activities in immunobiology, immunochemistry and immunogenetics in order to exploit new information and insights on immune system function and relevant applications to clinical problems and improved measures for diagnosis, treatment and prevention of immunologic and allergic diseases. Investigations on asthma, allergic mechanisms, immunologic diseases, biology of inflammation, immunopathology, transplantation biology and genetic associations have been especially promising. Some of the potentially rewarding areas that especially noteworthy and merit our attention are mentioned in this section.

It is anticipated that the basis of immunologic specificity, long a matter for speculation, soon will be more clearly understood as a result of ongoing studies in molecular genetics. Capacities for rearrangement and sequential expression of genes during differentiation of antibody producing cells likely are responsible for a large diversity of specificities among antibody molecules. Sophisticated instrumentation allowing for the dissection and molecular definition of the constant and hypervariable regions of antibody chains allows for such elucidation in studies with B lymphocytes. It is likely that similar investigative approaches can be rewarding in our ultimate understanding of the functional cellular specificity of T lymphocyte components.

The heterogeneity of antibodies produced in vivo in response to even the most highly purified antigens and the limited quantities of antibody reagents recovered as a result of immunization in suitable laboratory animals provide inherent difficulties in both experimental studies and the clinical use of immunologic reagents. Advent of the hybridoma technique whereby large amounts of uniquely and highly specific antibody molecules may be recovered by in vitro methods continues to be especially rewarding in overcoming these obstacles. We are only beginning to foresee how the availability of large libraries of monoclonal antibodies may be utilized in diagnosis and treatment of human diseases as well as in experimental studies.

In the laboratory these antibody reagents are being employed in the dissection of cellular interactions involving lymphocyte subsets and macrophages. In the clinical laboratory monoclonal antibodies can be expected to provide the most specific reagents available for pinpointed diagnostic studies utilizing immunoassay systems. Pathogenic features of a variety of heretofore poorly understood diseases now lend themselves to new definition and analysis; especially those arising on a putative basis of autoimmunity and aberrant immunologic reactivity, e.g. SLE, multiple sclerosis, juvenile diabetes, rheumatoid arthritis, granulomatous infectious processes, lymphomas and primary immunodeficiency disorders. In the area of therapeutic concepts, it is possible to now visualize specific drug tagged antibodies targeted at antigentically specific diseased cells and tissues. Methods have actually been devised to treat bone marrow of leukemic patients when in remission in order to remove traces of affected cells and allow for reinfusion following massive x-irradiation therapy during recurrences. Antibody products of hybridoma cells have also been used to monitor recipients of kidney transplants to obtain early indications of the onset of rejection and are being used to evaluate the action and utility of conventional drugs used to suppress the immune system in disease.

Another area of great potential offered by emerging advances in technology is that of the laboratory ability to clone immunocompetent cells. While cloning techniques for other types of body cells and bacteria have been well established early attempts to clone T and B cells were unsuccessful until several technical obstacles had been overcome. A major breakthrough has been the discovery and isolation of a T cell growth factor, interleukin, that enhances the proliferation of these cells outside the body.

The availability of lymphocyte clones could well improve approaches to the study of associations of human diseases with particular tissue (transplantation) antigens. In turn such information could lead to the ability to predict individuals at risk for certain diseases. With such knowledge appropriate preventive measures in turn could be developed and applied. It is possible that understanding cellular communication at the clonal level will allow for potential allergic and inflammatory reactions to be diverted to less harmful immune responses. Also, the availability of lymphocyte clones may make it possible to dissect the various types of immunodeficiency diseases and to apply this information to the diagnosis of autoimmune states. Although the clinical application of lymphocyte clones may seem years distant, one of the most promising clinical uses is in the treatment of cancer. It is believed that in some types of cancer failure of lymphocytes to attack and kill malignant cells may result from the inability of the tumor to activate potent lymphocyte killer cells. If such cells could be activated outside the body, then cloned and reinjected, elimination of the tumor could follow. Research in this area in early stages is promising. Similar applications of cloning can be envisioned to treat many other disease states where deficiencies and imbalances in regulation are identified, e.g., allergic disorders, virus infections, tuberculosis and parasitic infections.

It is appreciated that coordinated functions of the immune system in the maintenance of health require regulatory controls to effect appropriate immune responsiveness to antigenic stimulation. Deficiencies of constituent cells and their products and aberrant regulatory processes eventuate in immunologically mediated diseases. Accordingly, in order to successfully prevent, diagnose, control and treat immunologic and allergic diseases the need to continue and direct attention to studies concerned with immune regulatory control is merited.

Studies of immune regulation are proceeding at different levels. Included are definition of immunocyte membrane receptors, signals received by immunocyte receptors and affected by antigens, coordinated functions (circuits) of immunocyte intercellular communication by antibodies and secretory factors, and the total immune system reacting in a positive way to generate immunity or in a negative fashion resulting in tolerance. This concept of a large self-stimulating self-regulating unit established by a network of idiotypes is supported by data generated in ongoing research.

An increasing understanding of homeostatic mechanisms in immune regulation gives insight into amplification processes effected by selective cellular proliferation leading to lymphomatous processes, and conversely idiotype anti-idiotype interactions with resultant autoimmunity and immune complex pathologic features. Thus, the application of newly emerging technologies and instrumentation in molecular biology should permit a careful dissection of the molecular mechanisms controlling immunocompetent cellular interactions. In turn, we may be provided with the means to selectively manipulate the immune response for purposes of correcting human diseases resulting from disordered immune regulation. The development of highly sensitive, automated, protein microsequencer instrumentation, combined with recombinent DNA and hybridoma technology can provide the means for isolation and detailed structural and functional characterization of immunologically relevant proteins, such as recognition molecules on the surface of interacting cells.

The value of immunosuppression in treatment of a variety of autoimmune and immune complex mediated diseases and in preventing rejection of transplanted organs is well established. However, current immunosuppressive agents and modalities can be employed only with risk. Pharmacologic immunosuppression with corticosteroids and cytotoxic agents must be administered continuously following allografting and at least intermittently in long-term treatment of the immunologic diseases. Since the complications and dangers of prolonged use of these drugs in requisite dosages are appreciated alternative methods have been sought. Methods of potential promise include total lymphoid irradiation (TLI), cytopheresis and the use of monoclonal antibodies as discussed in an earlier section.

Methods for repetitive delivery of gamma-radiation to much of the lymphoid tissue of the body with protection of the lungs and marrow-containing bone originally had been developed for the treatment of Hodgkin's Disease and lymphomas. Resultant immunologic deficits indicating a predominance of suppressor mechanisms observed during the phase of recovery from TLI offer leads for possible use in organ transplantation. Cytopheresis, the continuous centrifugation of blood drawn from the patient to differentially sediment and separate blood cellular component, is applicable to removal of immunocompetent cells. Lymphocytes may be removed in large numbers and combined with plasmapheresis if removal of the antibody humoral component is also desired. Thus, a quicker and less cumbersome alternative to the concept of thoracic duct drainage can be feasibly visualized. In the case of monoclonal antibody developed for immunosuppressive anti-lymphocyte action, the objective is to selectively deplete specific helper cell subsets.

Exploitation of experimental leads developed in present day asthma and allergic disease research are important in helping us to understand heretofore unexplained abnormalities in bronchial reactivity and allergic inflammation beyond that effected by IgE mediated mechanisms. The search for explanations, especially in asthma is focusing on abnormalities in sympathic and parasympathic responsiveness and abnormal ratios between beta and alpha receptors. Also viewed is new information on the pathophysiologic effects of microbial antigens that enhance beta to alpha receptor conversion and the identification of anti-beta receptor antibodies in idiopathic asthma. Certainly an understanding of such mechanisms underlying abnormal findings could be the key to finding the basis of bronchial hyperreactivity and the design of specific pharmacologic agents to act on responsible pathways of adrenergic and cholinergic responsiveness.

New opportunities to treat and prevent asthma and allergic disorders stem from recent findings of research on the biochemical events involved in inflammatory reactions. Arachadonic acid, a fatty acid component of membrane phospholipides, has been demonstrated to play a key role in the production of the mediators of inflammation considered pathophysiologically important. Once freed from cell membrane through enzyme actions, arachadonic acid undergoes further metabolism along several defined pathways.

1. The cycloxygenase pathway responsible for production of several prostaglandins affecting blood vessels and central airways.

2. The lipoxygenase pathway resulting in a series of slow reacting substances of anaphylaxysis (SRS-A), now known as leucotrienes, capable of bronchoconstricting action on peripheral airways.

3. Platelet activating factor (PAF), a highly active lipid mediator of a spectrum of inflammatory and physiologic activities, perhaps one thousand times more potent than histamine in inducing vascular permeability and experimental anaphylaxis.

These discoveries relating to causes and mechanisms of inflammation in turn are leading to efforts in design and development of specific drugs to counteract ill effects and useful in the prevention and treatment of asthma and other allergic and immunologic disorders.

There is thus just reason to believe that effective drugs for successful management of the allergic patient can be put in the hands of clinicians in the not too distant future. In this newly developing era of immunopathology, relevance to the needs of the pharmaceutical chemist shows great promise. However, there still is just reason for appreciation of the role of specific treatment by immunotherapy in the management of the allergic patient. Clinical investigation is making it increasingly clear that an understanding of IgE generation and mast cell and eosinophil biology can offer opportunities to attack allergic reactions at definitive points of inception and in turn to successfully manage allergic patients by immunologically specific methods. Appropriately designed and controlled studies are helping to classify and define exactly those allergic disorders and types of patients than can be expected to respond to injection treatment. Accordingly, biochemical studies and assays of allergens constitute an important and progressive effort to place isolated, purified and well characterized allergenic fractions in the hands of the immunochemist engaged in clinically relevant investigations.

The lack of correlation between levels of induced serum blocking antibodies and clinical responses constitute the basis for elucidating other phenomena associated with clinical improvement following injection treatments. Under examination are the binding affinity of IgE to blocking antibody and the diminished capacity of affected basophils to release histamine following both specific and non-specific allergen challenges. Aimed at effecting more efficacious responses and less troublesome and involved plans for hyposensitization, ongoing work is designed to produce altered allergen preparations having either a slowed rate of absorption or an increased ratio of immunogenicity to allergenicity or both, e.g. by alum precipitation, formaldehyde treatment or gluteraldehyde polymerization of pollen extracts.

It has long been appreciated that even under the best of circumstances conventional injection treatment with allergenic extracts is only partially if at all successful in the majority of patients receiving immunotherapy. For newer approaches exploration of possibilities to induce tolerance rather than just IgG blocking antibodies is receiving increasing attention. The development and study of experimental animal models to search for factors of suppression of IgE function or inhibition of IgE formation shows much promise. Since it appears that the functional IgE-receptor is a unit and that IgE-receptor interactions are reversible, attempts to intervene in functioning of the unit may be possible.

By this approach and dependent upon the delineation of precise mechanisms of early steps in receptor-mediated activation, attempts could be directed to developing a specific inhibitor of the reaction.

There is also preliminary evidence that IgE antibody responses may be suppressed by inactivating precursor IgE B cells or by stimulating T regulator cells. These have included the administration of hapten-coupled non-immunogenic carriers to induce B cell tolerance and hence suppress IgE antibody formation, and the use of chemically modified antigens which do not react with antibodies against the native allergens but rather can maintain the ability to stimulate antigen-specific suppressor cells. Application of such studies to clinical investigation with modified allergens and allergen-carrier conjugates are underway.

Evidence that IgE antibody responses may also be regulated by nonspecific suppressor T cells similarly suggests possibilities for therapeutic intervention. Meriting a close look is experimental animal data demonstrating soluble factors derived from the T lymphocytes that differentially potentiate and suppress IgE responses. The presence of two factors that have affinity for IgE but opposite biologic functions can thus explain how the IgE response may be selectively regulated. If a similar suppressive factor can be obtained from human lymphocytes, its usefullness for therapeutic intervention in human allergic disease can be predicted.

Allergy and Clinical Immunology Branch

A. Scope

This Branch is concerned with the etiology, pathogenesis, diagnosis, prevention and treatment of allergic and immunologic diseases.

Studies of allergic diseases supported by the Branch include: (1) factors which contribute to asthma, such as extrinsic allergens, infections, abnormalities of the sympathetic nervous system, chemical mediators of inflammation, and pharmacologic agents; (2) immediate type hypersensitivity and its disorders (allergic rhinitis, atopic dermatitis, urticaria and angioedema); (3) allergic phenomena affecting respiratory, gastrointestinal and cutaneous tissues; (4) allergic disorders caused by insect bites and stings, foods, airborne allergens, and infectious agents; (5) immunoglobulins, particularly IgE, and the chemical mediators released by the interaction of antigen and antibody on target cells; (6) therapy and prevention of allergic disorders and hypersensitivity reactions; (7) manifestations of delayed hypersensitivity and contact dermatitis; (8) mechanisms of drug reactions and chemical sensitization; (9) isolation and chemical characterization of the active fractions of known allergenic agents; and (10) epidemiologic and environmental studies designed to ascertain those agents or substances which may be of clinical relevance to allergic individuals.

In the area of immunologic diseases, the Branch activities are focused on studies of the underlying mechanisms of disease and the application of basic knowledge to the etiology, prevention and management of immunologic disorders. Studies in clinical immunology are directed toward acquired and inherited diseases associated with dysfunctions of the immune system. Immunopathology studies include genetics, cytology, biochemistry, physiology and pharmacology of the immune system. Areas supported by the Branch include studies of (1) immune deficiency diseases arising from primary defects in development or disorders affecting immune responses, (2) clinical manifestations mediated by products of lymphocytes, (3) diseases associated with immune complexes and autoimmune phenomena, (4) immunodermatology, i.e., immune disorders involving the cutaneous system, and (5) immunotherapy of disease processes.

The ultimate goal of the Allergy and Clinical Immunology Branch is to promote the acquisition, translation, and application of research findings to the diagnosis, prevention, and treatment of allergic and immunologic diseases. To achieve this goal, the Branch is responsible for and manages Programs of Institute Emphasis (PIE) in Asthma and Allergic Diseases.

The immunologic disease centers and Program Projects are collaborative clinical and basic research studies supported in an effort to take advantage of the nationwide character of these programs and to facilitate achieving specific goals. In support of these efforts, the Branch funds contract efforts and also obtains and distributes selected research reagents and materials to investigative allergists and clinical immunologists.

B. Awards and Support Levels

ALLERGY AND CLINICAL IMMUNOLOGY BRANCH

FY 1981 AWARDS

AWARDS MECHANISMS	NUMBER	AMOUNT*
Research Grants	213	22,415,722.00
Career Awards and Career Development Awards	16	651,187.00
SUBTOTAL	229	23,066,909.00
Fellowship Awards	8	142,116.00
Training Awards	22	1,451,166.00
SUBTOTAL	30	1,593,282.00
CONTRACTS	8	137,875.00
TOTAL	267	24,798,066.00

* Total Costs, indirect costs estimated

The distribution of these awards by area of study is approximately 30% for asthma and allergic diseases and 70% for immunologic diseases. Approximately 34% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

Support for the PIE activities is included in the research grant category. During FY '81, the Branch supported these activities with awards for 17 Asthma and Allergic Diseases Centers at a total cost of $2,752,360, 7 Clinical Immunology and Immunopathology Program Projects at a total cost of $3,675,490 and 4 Centers for Interdisciplinary Research in Immunologic Diseases at a total cost of $1,256,944.

C. Program Areas and Highlights

1. Asthma and Allergic Diseases Centers Program (AADC)

A network of 17 Asthma and Allergic Disease Centers, located throughout the United States, is actively engaged in both fundamental and applied research. Through this multifaceted approach, methods have already been developed to improve the diagnosis and treatment of asthma and other allergic diseases. Although the emphasis of the Centers Program is directed toward a better understanding of the basic mechanisms involved in the various allergic disorders, they also have provided the field of allergy with unique academic resources for the discipline of allergy. By serving as referral centers for patients in their areas, engaging in collaborative clinical trials to assess diagnostic and therapeutic discoveries, and acting as an important resource for training academic allergists for the future, they have had a profound impact on the delivery of health care to allergic individuals. Separately, each of these Centers have also been successful in competing for additional support through either the regular grant program, training grants, or research career awards.

During the past year, two new centers whose primary investigation will be in dermatologic disorders were started, bringing to five the number of AADC Centers focusing on allergic dermatologic problems. One additional AADC was recently funded.

The 10th Annual AADC Workshop in Bethesda, on November 20-21, 1980 was expanded to include not only AADC representatives but also those from our Centers for Interdisciplinary Research in Immunologic Diseases (CIRIDs) (vide Infra) and Program Projects in Mechanisms of Immunologic Diseases (vide Infra). In addition, invited individuals from the lay community representing the Asthma and Allergy Foundation of America and the Allergy Professional Societies attended. About 125 participants met at NIH to consider not the progress in research during the proceeding year, but rather to explore collaborative efforts to promote the more rapid translation of research findings to the community and the bedside. As a consequence of this very successful meeting, efforts have begun to insure continuing outreach activities and to explore mutually beneficial community programs and other teaching methods, along with establishing a network of communication between Centers, NIAID and the community nationwide.

Brief highlights of some of the activities conducted by our Asthma and Allergic Disease Centers, not covered in last years report include:

Dr. Rebecca H. Buckley at the AADC at Duke University, grant #AI 12026, and her co-workers have continued their studies of the immunoregulation in atopic eczema. An important recent observation was a reaccessment of the conflicting data which have emerged from several laboratories regarding the kinetics of in vitro IgE production by human peripheral blood mononuclear cells. They have demonstrated that baseline quantities of IgE can not be accurately assessed by measuring IgE in day zero supernatants, since the main supernatant IgE concentration of cells frozen and thawed on day zero and incubated for seven to ten days were 3-10 fold higher. This slowly released IgE represented 40-50 percent of the peak quantities detected in cultures of atopic patients mononuclear cells, and 100% of those in normal controls. Overall their studies indicate that a majority of the incremental increase in the IgE by atopic mononuclear cells is contributed by a in vivo activated terminally differentiated cell which does not undergo replication and may be largely impervious to regulatory influences of other cell types of in vitro studies. Other studies on the regulation of IgE synthesis are currently underway. The data suggest that atopic patient's T-cells when compared with control T-cells have a diminished ability to suppress B-cell IgE synthesis.

Dr. Daniel Stechschulte at the University of Kansas Medical Center, grant #AI 15360, and coworkers have continued investigation in several important areas. A comparative study of Con-A nonspecific suppressor cell function and histamine receptor carrying mononuclear cells among atopic individuals showed no differences when compared with controls. The demonstration of mild dysfunction of histamine receptor carrying cells could be attributed to high levels of circulating histamine occurring in these individuals that might result in receptor modulation in vivo. In experiments comparing honeybee venom treated individuals to controls, results seemed to indicate that the most effective form of immunotherapy would be associated with the generation of a T-suppressor population for IgE synthesis in association with the generation of a T-helper cell population for IgG synthesis. Additional studies to confirm this are underway. Other studies have been carried out on bronchoalveolar cells from lung lavage. In in a preliminary series of experiments, SRS-A may be produced from blood mononuclear cells, but typically not from lavage cells, though simultaneously studied in the same individual. The inability of the lavage mononuclear cells to generate SRS-A maybe due to lack of a stimulatable synthetic pathway or to the rapid destruction of these molecules by the special mononuclear cells localized in the lung. Finally this group of investigators is conducting a clinical study to assess the efficacy of oral versus pulse prednisilone therapy in active lupus. It is too early to learn whether individuals can only be benefitted by daily therapy.

Dr. James Gilliam, grant #AI 17363, of the University of Texas Health Science Center at Dallas and his coworkers have been making an effort to characterize the contribution of epidermal Langerhan's cells to contact hypersensitivity. They have data that suggest that immunologic processes related to transplantation immunity and contact hypersensitivity are, in fact, interrelated, rather than totally independent, processes. Other studies have examined collagen antibodies in sera from patients with chronic inflammatory skin diseases, and used human mast cells from circumcised skin of male newborns to serve as a model of adult dermis mast cells.

Dr. Irma Gigli of the New York University grant #AI 17365, and her investigators have been able to isolate an IgE-like protein from the serum of a patient with scabies followed by pyoderma, who developed severe glomerulonephritis. This molecule seemed to act as an autoantibody similar to the C-3 nephritic factor of the alternative pathway of complement. It was also found in the serum from patients with lupus.

Other studies in this program have been aimed at examining human polymorphonuclear leukocyte function in patients with immune complexs or activated complement in the circulation; characterization of the soluble proteins of keratinocytes to determine whether tissue specific antigens of keratinocytes were retained by cells in culture; studies made to discover what causes the blistering observed in pemphigus.

Dr. Richard Hong at the University of Wisconsin grant #AI 10404, has continued to develop a guinea pig model of virus induced asthma using parainfluenza-3. Further studies on IgE synthesis continued. Preliminary studies suggested that IgE antibody formation may be the discriminating sign of adequate thymus reconstitution. Whereas most cytotoxic T-cell functions are restored with minimal amounts of thymus, much more tissue is required to acquire the capability for IgE production. Whether or not this observation can be utilized clinically will be the subject of continued observations. Finally, continued efforts to characterize beta-adrenergic receptor function are underway.

Dr. John B. Salvaggio, grant #AI 13401 of the Tulane University, and coworkers have continued their studies of mold sensitive and environmental asthma, and have accomplished the initial purification of Basidiomycete skin test materials. Clinical studies are now underway. Studies during the past year on tobacco smoke sensitivity have demonstrated among 93 individuals, half of whom claimed sensitivity to smoke, showed positive skin tests and RAST assays to extracts of tobacco leaf and tobacco smoke only in atopic individuals. They concluded that smoke sensitivity was not related to classic IgE mediated reactivity.

Dr. William L. Epstein, grant #AI 14752 at the University of California, San Francisco, his collaborators have been investigating immune mechanisms in atopic dermatitis. Family studies among Filipino individuals with severe atopic dermatitis demonstrated the combination of depressed cell mediated responses at markedly elevated IgE levels. Viral infections influence these responses adversely. Further studies of poison oak/ivy antigens (urushiol) are being made in an effort to begin a trial of testing whether or not this material is efficacious when given for desensitization to this contact dermatitis.

2. Centers for Interdisciplinary Research in Immunologic Diseases (CIRID)

In September 1978, 4 Centers for Interdisciplinary Research in Immunologic Diseases were funded by NIAID. This program is designed to foster integration and coordination of research projects in clinical immunology being pursued in clinical specialties (e.g., dermatology, pulmonary medicine, hematology, nephrology, rheumatology, infectious diseases and otorhinolaryngology) with those in basic research (e.g., immunochemistry, microbiology, virology, genetics, biochemistry, pharmacology, general physiology, and pathology). Furthermore, study of one or more allergic diseases is a necessary component. An important additional component of these Centers is the funding of specific projects in a variety of community activities which are designed essentially to impact on health care in a tangible, and immediate way. It is hoped that these efforts along with the traditional basic research components, may provide guidance and future direction in how to best accomplish this transfer of technology.

Dr. John Fahey grant #AI 15332 at UCLA, and his collaborators have made progress in several scientific areas. Human basophil desensitization was found to occur rapidly following the binding of IgE and then cross-linking by the addition of anti-IgE. Asthmatic subjects were found to have a selective loss of receptor responsiveness in large airways following inhalation of beta adrenergic bronchodilators. Presumably this is caused by abnormal air flow pattern with preferential deposition of aerosol particles in large airways. Protection against cytomegalovirus (CMV) infection in bone marrow transplant recipients was demonstrated with the administration of CMV immune plasma. Symptomatic CMV infections and interstitial pneumonia occurred less frequently in the group receiving plasma, indicating that passive immunization modifies human CMV infection and prevents interstitial pneumonia. These results are consistent with the hypothesis that latent CMV virus may be present in leukocytes of blood donors with previous CMV infections and after transfusion may be activated to produce active CMV infection. Because of these findings the prophylactic use of white cell transfusions has been discontinued at UCLA. Two new types of antibodies have been identified that react with A and B blood group antigens. Continued progress has been made with the modification of the childhood asthma curriculum, asthma care training (ACT), for education and involvement of children and parents in the management of the disease. Although followup is not complete, preliminary findings are encouraging in terms of decreased emergency room visits and lessened panic by children during asthmatic attacks. Additionally, the group has investigated a fascinating epidemic of cytomegalovirus and pneunocystis carinii pneumonia occurrances in homosexual patients which are thought to have depressed cell mediated immunity owing perhaps to chronic viral infections.

3. Program Projects in Mechanisms of Immunologic Diseases

Program Projects were established to encourage the development of collaborative basic science and clinical investigation in immunologic diseases. As such this program is designed to further investigate underlying mechanisms of disease and to enhance basic knowledge relevant to etiology, prevention and management of immunologic disorders. Clinical immunology studies are directed toward acquired and inherited diseases associated with dysfunctions of the immune system. Immunopathology studies include specific areas of genetics, cytology, biochemistry, physiology, and pharmacology of the immune system and its disorders.

Dr. Fred Rosen at the Children's Hospital Medical Center, grant #AI 05877, and his associates have continued a series of experiments relating to immunoregulation, particularly in children with immunodeficiency disorders. They have shown that individuals with congenital disorders of bone marrow functions can be successfully transplanted after receiving total body irradiation and rabbit antihuman thymocyte serum. These patients, including those with Wiscott-Aldrich Syndrome, infantile agranulocytosis and chronic granulomatous disease, have had successful lymphoid and hematopoietic replacement.

Studies here have shown that the cessation of acute graft versus host disease coincids with the appearance of peripheral blood T-cells with a suppressor phenotype, suggesting the disease is due to an imbalance between effector and suppressor lymphocytes.

They have further reported that patients with histiocytosis X have been successfully treated with thymic extract. The results were similar to historic controls, and the therapeutic outcome demonstrated that histiocytosis-X is not a neoplastic disease, but rather is a disorder of immunoregulation and that thymic hormone replacement therapy can be an effective form of treatment for some of these disorders.

Dr. David G. Marsh grant #AI 13370 of the Johns Hopkins University. Dr. Marsh and his coworkers have continued studies on the genetics of immune response in man. Their current hypothesis is that under immunogenetic limiting dosage exposure characteristic of inhalent allergy, the response to a specific antigen is regulated by relatively few specific immune response (IR), and immune suppressor (S) genes, including important loci linked to the HLA system. Further, they propose a generalized immune hyperfunction which regulates the overall synthesis of IgE antibodies and HLA associated genes which regulate immune responsiveness. Population studies have demonstrated that although males and females have similar reported rates of allergy, skin testing with a well defined panel of allergens revealed a significant higher prevalence of IgE mediated sensitivity in males when compared with females.

4. Mechanisms of Allergic Diseases

Studies within this program have been concerned with investigations of the etiologic factors, pathogenetic mechanisms, diagnostic measures, and therapeutic approaches related to the effective management of allergic disorders.

Dr. Byron B. Goldstein, grant #AI 16465, of the University of California, Los Alamos Science Lab, has continued work on a very interesting mathematical model aimed at understanding quantitatively the activation and desensitation of basophils by allergens. An early result is the answer to the question of whether heterogenicty is responsible for the bimodel histamine release curves occasionally reported from mast cell activation. Calculations from this group demonstrated that in general, bimodel binding affinity distribution do not give rise to bimodel cross-linking curves and therefore are not responsible for double humped histamine release curves. More likely this histamine relsease curve rises for certain IgE concentrations because the rate of histamine release saturates at a lower cross-linking concentration than the rate of desensitization. These investigators are now in the process of testing a current model of basophil activation and desensitization in which they propose a "calcium gating factor" which cross-link FC epsilon receptors to form a short lift calcium channel. After the channel forms the activating gating factor rapidely decays to an inactive form and it is the loss of the proposed active gating factor which causes the basophil to nonspecifically desensitize.

Dr. Andrew Saxon at UCLA, Grant #AI 15251, has extended studies of the regulation of human IgE in health and disease. They made an effort to determine whether or not the failure of regulation of IgE antibody synthesis might account for the abnormal persistence of IgE responses in allergic patients. By studying the synthesis of specific IgE and IgG in vitro in individuals allergic to honeybee venom undergoing venom immunotherapy, they were able to show that these allergc patients regulate specific IgE responses. An alternative explanation is therefore being sought to explain persistant elevations of IgE antibody.

Dr. John W. Yunginger grant #AI 16791 of the Mayo Foundation, has continued work on the chemical and clinical charterization of Alternaria allergens. Using a high volume air sampler, airborne particles were captured then immunochemically analyzed, permitting for the first time, data on atmospheric allergens which complements data obtained by traditional morphologic counts of pollen and spores. Estimates of the biologic activity of Alternaria-1 were made by using skin tests, intradermal end point titrations, as well as bronchial challenge techniques. Interestingly among 8 of 17 Alternaria sensitive individuals, positive bronchial challenge tests resulted at a crude Alternaria extract concentration between 500 and 5000 PNU. The Alternaria-1 immunotherapy study will permit the first controlled test of the clinical efficacy of mold immunotherapy using a defined standardized agent.

The work of Dr. E. Churchill McKinney at the University of Miami, grant #AI 16363, (New Investigator Award) concerns the characterization of an inhibitor of cell medicated immune reactions which has been derived from normal skin. Dr. McKinney is currently trying to test the hypothesis that the release of skin inhibitor material from damaged cells may actually function in vivo to limit inflammatory and immune responses and to promote the return of hemostasis. Release of this inhibitor during widespread cellular destruction could also depress reactions of circulating lymphocytes. Future work is aimed at attempting to demonstrate whether or not the skin inhibitor material may have regulatory function in the control of systemic cell mediated immune responses.

Dr. James Wedner of the Washington University, grant #AI 00441, (Research Career Development Award), during the time of this award was able to demonstrate a significant correlation between elevations of cyclic AMP and lymphocyte activation and to demonstrate that cyclic AMP is compartmentalized within the lymphocyte and only a specific cellular compartment, notably the plasma membrane, generated cyclic AMP in response to activating agents. He was subsequently able to demonstrate similar compartmentalization of protein kinase activity and to show the specific phosphorylation of a number of proteins associated with both the soluable and particulate fractions of lymphocytes in response to activating agents.

Dr. Jacob Pinnas of the University of Arizona in Tucson, grant #AI 16514, has successfully established colonies of Triatoma organisms and characterized the antigens involved in sensitization in humans. They are the so called "kissing bug bite" allergy. One or more of several salivary gland antigens in these animals may be responsible for the sensitization to "kissing bug" bites in each susceptible individual, and interestingly, among species, the antigens may not necessarily crossreact. Further efforts are therefore being conducted to find specific materials to study individuals and develop a rational approach to the diagnosis and treatment of this clinical allergy common in the southwestern United States.

Dr. Nickolas J. Calvanico at the Medical College of Wisconsin, grant #AI 157000, has been examining the allergens of Aspergillus fumigatus. An interesting observation was the discovery of a high percentage of marijuana smokers with Aspergillosis and the recognition of marijuana as a source of growth of the fungus. Therefore, marijuana smokers may be at risk to develop allergic bronchopulmonary Aspergillosis. Further studies are underway.

On grant #AI 16147, a new investigator award Dr. Wei Hsueh, of Northwestern University, has been studying prostoglandin biosynthesis among macrophages because of the role they play in the response of the lung to injurious agents. By comparing prostoglandin production by lung macrophages, they have shown that unlike peritoneal macrophages, they are able to secrete a wider spectrum of prostoglandin substances, and for example, in experimental granulomatous disease of the lung, there is a general reduction in prostoglandin release by macrophages in the resting state and during activation. However, a marked increase in the activity of lysosomal enzymes is found in these macrophages. Why these occur and whether clinical applications could utilize this is under investigation.

5. Asthma and the Other Allergic Diseases

Approximately 9 million Americans have asthma, a disorder of reversible airways obstruction. This obstruction has three major elements: 1) contraction of bronchial smooth muscles, 2) increased or retained secretions of mucous, and 3) inflammation of the respiratory tract mucosa. In individual asthmatics attacks can be provoked by exposure to specific allergens, and some of these individuals may have other allergies such as allergic rhinitis. On the other hand, many asthmatics do not have a demonstrable allergy, and in these individuals a variety of infectious, nervous (adrenergic and cholinergic), genetic or inflammatory factors may play a primary predisposing or contributory role. The program on asthma is structured to investigate all of the major parameters contributing to describing the mechanisms of causation in this disease. Areas under investigation include: the natural history, allergens, broncho-provocation challenge, animal models, pulmonary function testing, chemical mediators, cell mediators, adrenergic agonists and antagonists, parasympathomedic agents, drug allergy, diagnostic methodology, and therapeutic agents.

Dr. Miles Weinberger at the University of Iowa grant # AI 16151 has been developing an effective, safe and teachable program for the management of children with asthma. Physicians need to learn to use currently available medications in the best possible fashion. Oral theophylline therapy has been compared with metaproterenol among 34 children treated in a randomized manner with the two drugs. Theophylline correctly used was associated with a 50% greater frequency of totally asymptomatic days, and resulted in improved exercise tolerance, higher peak flows, and only 1/10 the risk that symptoms would become unresponsive to bronchodilators thus requiring the additional use of prednisone. Other studies include: an investigation of steroids or inhaled Beclomethasone diproprionate. Growth suppression among these individuals appeared due to the disease rather than to a drug effect per se. Moreover this group continued to find that inhaled steroids are not free from side effects.

On training grant #T32 AI 07007 of Dr. Alan Winklestein of the Johns Hopkins University School of Medicine, Dr. Hailen Mak has, in collaboration with American Lung Association of Maryland, surveyed over 10,000 first and sixth graders in Maryland public schools to determine the prevalence of asthma, to determine the extent of school absenteeism due to chronic asthma, and to examine the pattern of health service utilization by asthmatic children in the State of Maryland. They have found that the cumulative prevalence of asthma was 10.5% for Baltimore City and 8% for the rest of Maryland. The prevalence was significantly higher for males, the male/female ratio being 1.6 to 1. and for blacks, the black/white ratio being 1.5 to 1. Nearly 50% of the children with active asthma in Baltimore City missed six days or more out of the school year because of illnesses due to asthma. Almost half of the asthmatic children in Baltimore obtained their care of asthma in an emergency room and twice as many blacks as well as whites used an emergency room as their primary source of care. Moreover, emergency room users had a higher school absentee rate than nonemergency room users. On the bases of this educational programs and health service efforts are currently being planned.

Dr. Oscar L. Frick, grant #AI 15233, at the University of California, San Francisco, has made interesting progress in the outbred hunting dog model of the development of asthma. After identifying high skin reactivity animals, young puppies were given live distemper vaccine followed by the injection of inhalation of pollen extracts. This resulted in 2/3 of the animals becoming high IgE antibody producers to pollens and about 10% of the animals developed eczema at certain times correlated the pollen seasons. More recently upon inhalation challenge with ragweed pollen, six dogs demonstrated bronchospasm as measured by changes in flow volumne curves. Although not too many animals have been bred, this would represent a successful use of the dog as an animal model for airways bronchospasm, although the pollen allergy is artifically induced in these animals. Perhaps more importantly, the animals also provide a model of dermatitis.

Training grant #AI 07166 of Dr. Henry Claman at the University of Colorado has as one of the trainees, Dr. Portanova, who is studying types of antibodies produced by patients taking procainamide and hydralazine. These drugs can cause the appearance of autoantibody and it has been demonstrated that antibodies to histones, chemicals normally present in nuclei, are made by patients on these drugs. However, the anti-histone antibodies made after taking hydralazine are different from those made after taking procainamide. Studies like this should be helpful in developing diagnostic tests for drug allergies.

Dr. Robert E. Reisman, grant #AI 14501, at the State University of New York at Buffalo, has continued studies of insect allergy confirming previous suspicions that individuals who have experienced only large local reactions do not require venom immunotherapy, irrespective of their immune status. There finding was that subsequent stings usually lead to further local reactions but no systemic reactions. They have additionally shown that adequate venom immunization may be done in a "rush" method with subsequent injections given within the period of six to eight weeks to reach maintenance dose.

Dr. William R. Soloman at the University of Michigan-Ann Arbor, grant #AI 10181, has continued studies of aeroallergens. They found that homes with ten or more house plants did not necessarily contain unexpectedly high fungal spore levels. By disturbing the plants there were some increases in the levels of penicillin species, but in general plants were not considered a major source of airborne allergic material. Importantly they were unable to confirm European reports of an elevated number of Aspergillus fumigatus levels associated with house plants. Further studies in a botanical garden established that spore levels in the very humid plant filled interiors were in fact high, and increased significantly during watering. Aspergillus was not frequent nor abundant in those greenhouses.

Dr. Kenneth Kranning, grant # AI 16636, at the University of Washington, has been examining sweating responses in normal and allergic individuals. Since sweat glands exhibit many of the properties of less accessable organs and tissues, for example adrenergic and colonergic receptors and a nervous inovation from the anatomic nervous system, one could use them to examine defects potentially described in pharmacologic responsiveness such as that postualted beta adrenergic defect in atopic disease. Therefore, Dr. Kranning is making an effort to utilize the sweat gland has a potential diagnostic indicator of atopic disease.

For Dr. Kurt J. Block, Training Grant #T32 AI 07040 at the Massachusetts General Hospital, one area of investigation has been that of photo immunobiology. Because ultraviolet radiation in combination with psoralens is widely used in the treatment of psoriasis, and because the UV light penetrates the skin to a depth of several milimeters, lymphocytes passing through the lymphatics are irradiated. They have demonstrated that the treatment of psoriatic patients leads to changes in the peripheral blood lymphocyte distribution and function which are reversible during a single course of treatment. However, in persons maintained on this therapy for four years persistant changes were observed, which consisted of a reduction in the percentage of helper induced T lymphocytes and the loss of responsiveness to mitogens. Two individuals who had the most marked abnormality in these measures developed skin cancer. Whether or not this treatment should be eliminated in patients with persistent lymphocytes abnormalities is under study at this time.

Under a Fellowship grant #AI 06432 in conjunction with Dr. David Katz, Dr. Fu Tong Liu at the Scripps Clinic is working to develop DGL conjugates to allergens to produce abrogation of immune response in allergic individuals. For example, they have previously demonstrated that conjugates of complexes protein molecules and a synthetic polymer of D-glutamic acid and D-lysine (DGL) could induce an immunologic unresponsiveness. They conjugated antigen-E, the major allergen present in ragweed, to DGL and then injected this conjugate into either unimmunized mice or mice that were immunized with antigen-E. The animals were no longer able to make an anit-antigen-E response. If this can be accomplished in man, the protein-DGL conjugates could be of great value as therapeutic agents in the treatment of allergic disease.

Dr. Stephen I Wasserman, an Academic Allergic Disease awardee grant, #KO7 AI 00431, and a regular grant awardee, grant #AI 17268, of the University of California, San Diego, continues his studies of the release and effect of the mast cell and basophil mediators in human disease. Three principal mediators, differing in size, charge and hydrophobic nature have been isolated. Each is chemotactic for human eosinophils and deactivates this cell. The two size categories the mediators fall into are: 1) molecular weight 300-700, which is highly acidic, hydrophobic and resembles the eosinophil chemotactic factor of anaphylaxis; 2) molecular weight 1-3000, which are less acidic and less hydrophobic. In addition, a high molecular weight neutrophil chemotactic factor has been demonstrated, has been partially purified and appears to be identical to the high neutrophil chemotactic factor generated in cold urticaria.

Dr. Fred S. Kantor, grant #AI 17716, of Yale University, and his associates have initiated experiments designed to examine the role of auto-antiidiotypic immunity in the regulation of immune responses in animal models of human disease. They have induced experimental autoimmune myasthenia gravis in C/57 black mice and then correlated the cellular immune response and the development of anti-acetylcholine receptor antibody. The cellular immune response was found to peak in about the second week after immunization, remaining at a high level until the fourth or fifth week. The antibody titer, on the other hand, rises in the second week and remained high throughout the eight weeks of the study. The electrophysiologic changes follow shortly after the antibody titer curve begins to stay up. Finally, clinical disease did not appear until the fifth or sixth week increasing through the tenth week. They now propose to continue efforts to determine which element of the immune response is actually paramount in the development of the clinical disease. These studies will help us understand how humans acquire myasthenia gravis and thereby determine appropriate treatment.

Dr. Burton Zweiman at the University of Pennsylvania grant #AI 14332, has continued studies of the inflammatory responses and allergic reactions finding that the local application of terbutaline, a beta adrenergic agonist, inhibits both the release of histamine and inflammatory cell responses in human skin allergic reactions to pollen antigens. This and other studies he is conducting are important in unraveling the approach to pharmacologic therapy of atopic skin disorders.

Dr. Robert A. Lewis, an Academic Allergic Disease awardee, grant #AI 00399 at the Robert B. Brigham Hospital, has continued studying the regulation of mast cell-associated mediator release. For example, it has been shown that the interaction of human lung mast cells, and allergic antibodies caused a release of an airway constricting, blood vessel dilating product, prostoglandin D-2 from a component of mast cell membranes, suggesting that interference with mast cell granule secretion might not be sufficient to prevent certain clinical aspects of allergic disease. Indeed, in a nonallergic disease of mast cell proliferation, mastocytosis, Dr. Lewis has demonstrated urinary metabolites of prostoglandin D-2 suggesting that therapy directed against the synthesis of prostoglandin D-2 could help in preventing the manifestations of the disorder. Dr. Lewis has demonstrated that leukotriene B-4, which is believed to attract white cells into areas of inflammation, were structurally identified, and the naturally recurring compound was shown not only capable of attracting white blood cells in a laboratory model system, but also in the skins of monkey and humans, thus confirming its purported biological effect.

Interesting studies have been conducted under the Training Grant of Dr. H. Hugh Fudenberg at the Medical University of South Carolina, grant #AI 07063. Dr. Mathur of that group has conducted investigation of the role of the immune system in fertility and infertility, and developed two assay systems for measuring immunity to sperm antigens in males and females. She has recently demonstrated that many couples with unexplained infertility have in their circulation antibodies that react with sperm cells. After treatment with prednisone, which reduced the anti-sperm antibody level, three of six previously infertile couples were able to achieve pregnancy.

6. Immunodeficiency

The program on immunodeficiency is concerned with the etiology, ontogeny, prevention, and treatment of structural and functional deficiencies of the immune system. Both naturally occurring and acquired disease states are included. Ongoing studies have focused on the congenital absence, failure of development, or other disorders affecting thymus or bone marrow cellular elements; abnormalities in production, inhibition or catabolism of immunoglobulin; deficiencies of specific complement components; and defective host defenses due to abnormalities of leukocytes. These naturally occurring or acquired defects of immunity provide a unique opportunity to expand the understanding of normal immune function. Only by its absence do we see a role for its presence in the sequence of steps which make up the normal immune response. Although these diseases are rare, the information gained is relevant to the general field of immunology and to many health problems including infectious diseases, allergy and arthritis.

Dr. Ronald H. Stevens of the UCLA, grant #AI 16694, has continued studies examining humoral immune regulation in immune deficiency showing that the majority of patients with common variable immunodeficiency failed to generate a distinct population of B-cells, which is possibly the earliest B-cell subset to appear in the periphery following immunization. They also have developed a model of antibody deficiency in normals whereby a transient state of unresponsiveness is induced by a repeated antigen exposure, thus permitting additional experiments.

Dr. Jerald Schwaber of the Children's Hospital Medical Center, grant #AI 16731, under the auspices of a New Investigator Award, has been studying B-lymphocytes in X-linked agammaglobulinanemia. A finding of interest has been a direct correlation between the subsets of B-lymphocytes present in the peripherial blood of immunodeficient individuals and the isotypes expressed by B-cell lines derived from these individuals. His results further strengthen the argument that agammaglobulinanemia results from a failure of the B-lymphocyte.

Dr. Irving Goldschneider at the University of Connecticut, grant #AI 09649, in the course of continuing studies concerning the development of immunologically competent lymphocytes has studied the expression of three nucleotide-metabolizing enzymes during T-cell development. Since inherited deficiencies of these enzymes have been associated with immunodeficiency disorders in humans, these studies are expected to shed light on such deficiencies.

In short, the results suggests that specific stages of T-cell development may be characterized by the absolute and relative levels of various enzymes. Further, that selective deficiencies of these enzymes might induce immunodeficiency disorders by affecting discreet stages of T-cell differentiation. Such knowledge is important before one can exert a therapeutic or interventive influence in patients.

Dr. David B. Van Wyck a New Investigator Awardee at the University of Arizona grant #AI 16926, has been studying the use of partial splenectomy and its consequences. Because of the serious and frequently fatal blood stream infections suffered after individuals have undergone splenectomy, often highest among children, alternatives to splenectomy which leave behind a small splenic remnant, have been developed to restore or retain the spleen's capacity to resist infection. Interestingly, he has been able to introduce a splenic artery embolism through a small catheter which can effectively reduce splenic mass and control splenic hyperfunction.

Dr. Peter Jezyk, grant #AI 17791 at the University of Pennsylvania, has extended efforts to expand the breeding stock of a colony of dogs with an inherited variable combined immunodeficiency in order to produce an animal model for further investigation of this disease which appears comparable to that seen in humans.

Dr. David Terman at the Baylor College of Medicine, a recipient of a Research Career Development Award, grant #AI 00302, has continued research examining extracorporeal immunoabsorbents for the treatment of neoplastic and autoimmune disease. During the past year he had begun a clinical trial employing protein-A collodion-charcoal (PACC) in patients with advanced breast cancer. He found that perfusion of small volumnes of autologous or homologous plasma in the amount of 100-300 millimeters in patients with breast cancer resulted in rapid necrolytic reactions localized to tumor sites. Controlled plasma from normal donors produced no results. Treatment could be repeated at intervals of every second day with no untoward clinical effects. This method promises to shed new light on the therapy of cancer.

Other studies have shown that PACC can generate cytotoxic tumor specific antibodies. By isolating and purifying this tumor specific material from tumor bearing plasma it is hoped to produce this substance in the laboratory. Initial efforts are being directed at isolating this tumoricidal factor from the serum of advanced breast careinoma; patients with advanced breast and administering purified or partially purified substance ultimately to humans.

Dr. Dennis Ownby, grant #AI 17741 at the Henry Ford Hospital, has been studying adenosine deaminase levels because of its known association with human immune dysfunction. Screening patients disclosed one individual with severe combined immunodeficiency. Further studies showed the absence of an enzyme called adenosyl homocysteine hydrolase in this person. Biochemical differences in subclasses of lymphocytes which may be associated with immunodeficiency diseases is expected to lead to enhanced diagnosis, and therapy.

7. Immune Complexes Disease

Immune complexes result from the binding of antigens by antibodies. Subsequently, components of the complement system also become involved. Such complexes, therefore, arise during the course of the development of the normal immune response. Under some circumstances, however, these entities appear within lesions and indeed are necessary for the development. The pathogenetic mechanisms by which immune compelxes cause tissue damage, particularly in the auto-immune diseases with which they are increasingly associated assays for types of immune complexes has impeded work in this area to some extent.

Dr. Raphael J. De Horatius, grant #AI 14574, with a New Investigator Award at the Thomas Jefferson University, has examined aspects of immune regulations in lupus, specifically the effect of plasmapheresis on immune regulation. Results from this Institution are at variance with those from Stoney Brook and Allan Kaplan. Horatius has shown that plasmapheresis in a small group of patients was detrimental, and resulted in an excerbation of their disease manifested by worsening of renal involvement in two, and worsening of thrombocitopenia in a third. Two of the four patients had a rise in circulating immune complexes post-plasmapheresis which probably contributed to their disease exacerbation.

Dr. Norman Talal, training grant #AI 15550, of the University of Califronia, San Francisco, and his coworkers have continued studying systemic lupus erythematosis (SLE) models in inbred strains of mice, demonstrating that male and female sex hormones greatly influence the expression of murine lupus in an older animal model. The male hormone suppresses all aspects of the disease and prolongs life. These studies provide some explanation of why lupus occurs predominantly in women. Studies more recently have shown the protective effect of male hormone in two new mouse strains. Further, a deficiency of another type of hormone in the mice manifesting lupus has been found, a hormone called interleukin-2 or T-cell growth factor. Male hormones preserve interleukin-2 activity whic may explain its protective effect.

Dr. Frank Fitch at the University of Chicago, investigating mechanisms which enhance survival of kidney transplants, grant #AI 09268, has determined that anti-idiotypic antibody produces selective suppression of rat allograft rejection, after injection of alloantigen and alloantibody. It is known for example that kidney allografts in the rat survive indefinately if the recipient is treated before grafting with donor antigens and with anti-donor antibody. By using specific antibodies, various levels of suppression can be accomplished.

Dr. Alfred F. Michael at the University of Minnesota, grant #AI 10704, in addition to conducting basic research, has embarked on a outreach demonstration program to educate the community of the importance of biomedical research with respect to kidney disease in children. They have published a small booklet entitled "Something Special for Distribution To Children who Have Kidney Disease", the booklet is intended to act as a prototype for communicating directly to the sick child and his or her family.

8. Inflammation

The process of inflammation is mediated by cells and by humoral factors. This process is essential for host defense against foreign substances. However, in certain disease states, inflammation plays a major role in tissue damage. An understanding of what initiates and what modulates inflammation is, therefore, crucial to explaining and controlling these disease states. The noncellular mediators of inflammation can be divided into two general categories, those of small molecular weight and those of high molecular weight. The group of serum proteins derived from the complement system and the associated enzyme systems of coagulation, fibrinolysis, and kinin fall into the latter category.

The initiation of the complement cascade by either the classic or alternate pathway makes a major contribution to the development of the inflammatory response. Once activated, complement components have functions including opsonization, immune adherence, chemotaxis, cell lysis, and anaphylaxin generation. Derangements of complement have been implicated in causing or contributing to a wide range of disorders including cancer, kidney diseases, collagen vascular diseases, infectious diseases, and inherited diseases. Understanding the complement system depends on isolation and identification of its components. It is known that there are approximately 20 complement components.

Dr. Alfred I. Tauber, grant #AI 15116, a new Investigator Awardee from the Robert B. Brigham Hospital, has been studying granulocyte function. Since the bacteriocidal capacity of the human neutrophil is the the first line of defense against microbes and is dependent on the cells ability to generate the respiratory burst, causing a marked increase in oxygen consumption by the activated cell, and the formation of several highly reactive and potentially lethal free radicals. These metabolites of oxygen, superoxide and the hydroxy radical, are not only potentially damaging to invading pathogens but may cause damage to host tissues and to the cells responsible for their production. Dr. Tauber has isolated the respiratory burst enzyme, and partially characterized its biochemical and physical properties. The elucidation of the molecular formation of superoxide by the respiratory burst enzyme is a major advance in the understanding of the normal function of neutrophil bacteriocidal mechanisms. The new findings that the oxidase activity is activated by phagocytosis, but that superoxide is not necessarily produced is an important observation.

Other investigations in this area include Dr. William R. Henderson, Jr. at the University of Washington, grant #AI 14805. He has demonstrated that mast cells are able to kill tumor cells. The mast cell induced tumor cell killing was caused by an enzyme, peroxidase, which is found in granules within the mass cells and released onto the tumor cell's surface when mast cells are stimulated to release their granule contents, and hydrogen peroxide. He has also found that esoinophils can produce leukotrienes and further that peroxidase and hydrogen peroxide released by stimulated eosinophils can degrade the newly released leukotrienes. Studies of the release of mediators as well as enzymes from various cells in both inflammatory and allergic reactions are of extreme importance.

Dr. J. Dwight Stinnett, grant #AI 15675, of the University of Cincinnati has been performing a series of studies aimed at identifying subpopulations of neutrophils. To date, he has shown at least two populations of polymorphonuclearleukocytes, adherent and nonadherent, determined by attraction to lectin columns. Differences demonstrated include finding that the nonadherent population exhibits a poor chemotactic response to complement.

Dr. Richard K. Root at Yale University, grant #AI 13251, and his collaborators have continued efforts to define the mechanisms responsible for the normal microbicidal functions of human phagocytic leukocytes and their abnormalities in individuals who had increased succeptibility for infection. In the course of such studies, they have demonstrated a lack of myeloperoxidase, an enzyme thought to play a major role in the ability of white blood cells to kill bacteria and fungi, to be missing from neutrophils and monocytes of a large number of susceptible individuals. More importantly, the combination of diabetes mellitus along with this enzyme deficiency may result in severe fungal infections in some individuals.

Dr. Samuel C. Silverstein of the Rockefeller University, grant #AI 08697, during the course of studies on the role of mononuclear leukocytes in immunity, was able to demonstrate that the Legionnaire's Disease bacterium (Legionella pneumophila) occurred more frequently in immunosuppressed patients. They established that the bacillus grows intracellularly in human monocytes, and further that the Legionnaire's Disease bacillus is refractory to lysis by antibody and complement and is resistant to killing by polymorphonuclear leukocytes and monocytes. Even when coated with antibodies and complement, this bacillus grows in human monocytes at the same rate and to the same extent as unopsonized bacteria. These findings indicate that humoral immunity may play little or no role in protection against this disease.

Dr. Robert Webster, grant #AI 05971, at the National Jewish Hospital and Research Center, has been studying the biologic properties of human complement component, C5a. Because neutrophils accumulating in the air spaces of the lung appeared to be a common event in many lung diseases, there has been a special effort to understand the mechanism by which neutorphils accumulate in lung infiltrative processes. Furthermore, recent evidence has suggested that the activation of the complement system which generates biologically potent fragments may play a major role in the cause of these conditions. It appears that the fragments from C5 play an important role in producing lung injury and further studies are underway to elucidate this mechanism.

Dr. John R. David of the Robert B. Brigham Hospital, grant #AI 07685, has continued studies in cellular immunity, purifying the lymphocyte mediator, MIF. Their studies of collagen immunology have been directed to find out how autologous cartilage might induce an immunologic reaction to collagen. Of special interest is the finding that if cells to which collagen has been bound are injected in a certain way, they have suppressed the induction of immunity to collagen. Overall these efforts are expected to help determine whether suppression of immunity to collagen might be therapeutic for arthritic or collagen disorders in man.

Genetics and Transplantation Biology Program

A. Scope

This Branch supports and manages research on the immunologic and genetic factors that determine the acceptance or rejection of grafts.

The increasing prevalence of degenerative disease and of trauma that may impair the function of particular organs sufficiently to be incapacitating or lifethreatening identifies the perfection of a successful transplantation methodology as one of the objectives of highest priority in current medical science. The technical surgical aspects have proved quite manageable but immunological rejection of the graft is the obstacle that has yet to be surmounted.

Research efforts supported by this Branch include:

1. Elucidation of the structure, organization and control of the expression of genes on which immunological activity depends.
2. Studies of strategies designed to minimize the likelihood of rejection by assuring the best possible antigenic match between donor and recipient.
3. Explorations of immunosuppressive regimens whose objective is to reduce the capacity of the host to reject the graft.
4. Studies of treatments of the graft that will reduce its ability to provoke a response.

Studies in animal models are supported that explore the genetic controls of the immune response, and, on a more practical level, test methodologies whose ultimate application is to man.

Genetically conditioned associations between antigenic characteristics and susceptibility to various diseases have recently been brought to light and also fall within the purview of this Branch.

The Serum Bank is an important distinct component of the Branch. It is an international resource, providing sera and homozygous typing cells for research on human immunogenetics and for histocompatibility testing performed in conjunction with organ transplantation. The continuing evolution of the Bank is currently taking it into information gathering and analysis in the field of population immunogenetics.

B. Awards and Support Levels

The Genetics and Transplantation Biology Branch supports research activities through individual grants, research career development awards, fellowships, training grants, and very importantly, in connection with its organ transplant program and serum banks, through contracts.

The following shows the distribution of support for the activities of the Branch during FY 1980.

Genetics and Transplantation Biology Branch
FY '81 Award

Award Mechanism	Number	Amount*
Research Grants	81	$ 8,712,375
Career and Career Development Awards	9	319,203
Subtotal	90	$ 9,031,578
Fellowship Awards	7	123,904
Training Awards	1	22,703
Subtotal	8	$ 146,607
Contracts	26	$ 1,289,619
Total	124	$10,467,804

* Total Costs, indirect cost estimated

The majority of these awards are concerned directly with transplantation biology and the remainder, a rapidly increasing component, support research on the genetics of the immune system in general and of susceptibility to disease, in particular. Approximately 30% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

C. Program Highlights

1. Transplantation

(a) The Development of Statistical Model To Assist Decision Making in Transplantation

A continuing major undertaking of the Genetics and Transplantation Biology Branch is the analysis of data on renal transplantation in an attempt to identify those factors that favorably or adversely affect outcomes. For that purpose three large data bases are being investigated (a) the Kidney Transplant Histocompatibility Study (b) the Medical Information System of the End Stage Renal Disease Program, HCFA, DHHS, which contains information on nearly all persons on dialysis or who have received renal transplants, and (c) the data of the South-East Organ Procurement Foundation (SEOPF).

These efforts find their justification both in medical and in economic considerations. In 1979 there were about 45,000 persons on dialysis, a number that is projected to rise by 9,000 per year. The average overall cost of dialysis was $28,000 per patient per year in 1979 and probably approaches $40,000 now. Somewhat more than 4,000 transplants are performed per year in the U.S. The cost in the first year, including the transplantation is about $29,000, and if the graft continues to function, drops to about $1,000 per year in the subsequent-years. As about half the patients on dialysis are likely to benefit from transplantation, it is clear that enhancing the success of the procedure is highly desirable not only from a medical but also from an economic point of view.

The NIAID KTHS was a large scale prospective observational study whose overall objective was to identify those factors that affect, whether favorably or unfavorably, the outcome of renal transplantation in terms of the survival of the recipient (patient survival), retention of a functioning graft (graft survival), and of restoration of the recipient of the graft to a normal life. Over 40 centers participated in the study. The transplants were performed in 1974-1976 and followup continued until the end of 1978. A total of 2410 patients were studied, of whom 885 received transplants from living related donors and 1533 from unrelated persons who had died suddenly, usually as a result of head injuries, but also, in some, cases as a result of a cardio- or cerebro-vascular accident or some other even less frequent causes. There was a disproportionately high frequency of black recipients of cadaveric transplants (20%). The recipients of living donor grafts tended to be younger than the recipients of cadaveric grafts. Among the identified causes of renal failure, glomerulonephritis clearly predominates, with diabetes, hypertension and pyelonephritis also major causes. Malignant hypertension and diabetes as causes of renal failure have distinctly different racial distributions. The incidence of malignant hypertension in the blacks in the study was 32% and 5% in the whites. Diabetic glomerulopathy, on the other hand, was found 3.5 times more often in whites, 14% vs 4%.

Grafting of kidneys from living related donors has results that are much superior to the transplantation of cadaveric kidneys. For example, one year after implantation, 75% of the former are still functioning compared to only 49% of the latter (the standard errors of the estimates are +1-2%). There is also a difference in mortality rates. Again at one year, 90% of the recipients of living-donor grafts are alive vs 81% of the recipients of cadaveric grafts. One additional statistic must be mentioned here, that which describes the efficacy of transplantation in rehabilitating the recipient. Of those whose grafts are still functioning after one year, 94% of the recipients of living donor grafts and 89% of the recipients of cadaveric grafts are engaged in full-time activity, i.e. are not significantly impaired in their ability to function in the world.

The differences in the survival of living donor and cadaveric donor grafts point to major biological factors that determine the long-term outcome of transplantation. The identification and the assignment of relative weights to these factors to permit their use as guides to practice requires the application of the techniques of multivariate analysis. A statistical model was developed by Bailey, Homer and Summe of the Naval Medical Research Institute specifically for this purpose.

The probability of graft retention to any point in time is determined, in this model, in terms of quantities interpretable as the excess initial risk, the rate of decays of this risk, and the long-term risk. The contributions of the risk-factors to each of these quantities can be considered their weights or measures of their relative importance.

The data collected in the KTHS Study were used to evaluate the weights associated with risk factors such as age, race, antigen match and various others and to estimate the probability that the observed effect of the putative risk factor is due to chance. The living donor and the cadaveric donor transplants were subjected to this type of analysis in terms of 13 risk factors. These include, for example for the cadaveric donor: age of recipient, race of recipient, diabetes as primary disease, hypertension as primary disease, pretransplant transfusions, perioperative transfusions, number of prior transplants, pretransplant splenectomy, preformed antibodies, ATG prophylaxis, serological mismatch, cold ischemia time, major center effect. Once the weights of these factors are available, it becomes a very easy matter to predict the probability that a transplanted kidney will continue to function in any real or hypothetical recipient up to any specified point in time. This ability to provide an objective, quantitative estimate of the probability of graft survival in various potential recipients is the most important result of the KTH Study because it should permit the stratification of qualified recipients and therefore the attainment of higher success rates in transplantation. One must of course beware: the prediction is no more valid or accurate than the weights computed for the risk factors, and these in turn are only as valid as the data that were collected in the KTH Study. To this point in time, that study is the most searching and comprehensive available and though perfection was not attained, very great efforts were made to ensure the accuracy of the data.

A few examples will illustrate how the predictive model may be used as a guide to practice. In the living-related donor transplant case, analysis clearly demonstrates that the most important predictors of graft survival are the serological haplotype match and the cellular crossmatch. If recipients of cadaveric transplants are compared with equivalent recipients of living donor transplants in which donor and recipient are zero or one-haplotype matched and reactive in the cellular crossmatch, it is found that the graft survival at one year (70±8%) is not sufficiently different in the living donor case from the cadaveric donor case (64±4%) to justify the use of a living donor. On the other hand, a well matched living donor transplant has truly excellent prospects (94±1%).

Cadaveric kidneys are often transported substantial distances to where a recipient awaits. Waiting lists for organs are, in general long. The choice of the recipient who is most appropriate is, of course, difficult and based on a multitude of factors, one of which must, clearly, be the probability of success. Because prolonged storage time of the kidney is associated with decreased graft survival, a tradeoff between a recipient with a poorer prognosis but who is nearby with a more distant one may be necessary. Here also, quantitative estimates are possible. Age is associated with a reduced graft survival. But a 45 year old recipient who can be transplanted within 10 hours of salvage of the cadaveric kidney is on a par with a 25 year old for whom a delay of 45 hours would be required.

This model may also be used as a guide to the choice of therapies. For example, for otherwise comparable persons, omission of pretransplant transfusions reduces the 1-year graft survival from 64±4% to 58±5%, while use of antilymphocyte serum in the initial program of immunosuppression raises it to 68 ±5%.

These data are at this time very readily accessed. The program for prediction of outcomes can be readily written for the more capable hand held calculators. Alternatively, if we at NIAID are provided with the characteristics of the patients to be transplanted, predicted graft survivals can be made available to the inquirer within about one-half hour.

The application of the statistical model as a guide to practice must at this time be tentative and cautious because the weights of the risk factors were deduced from but one data base which refers to practices employed some years ago. Consequently, a collaboration has been undertaken with Drs. William Vaughn and Everett K. Spees, Jr. representing the South-East Organ Procurement Foundation. That organization has accumulated extensive information on cadaveric donor renal transplants performed from 1978 on in the south eastern U.S, about 1600 in all. The weights of nine risk factors were evaluated in parallel for the two data bases by means of the above described statistical model, and then were used to predict outcomes for model transplant recipients. The predictions for the short term of the two sets of risk factor weights are quite similar, with some exceptions to be described below. One extremely valuable lesson about statistical analyses of patients requiring long-term follow-up was learned immediately. In the KTHS, the minimum follow-up was 2 years and the maximum five years. In the continuing SEOPF study, the follow-up on many patients is less than one year and, because of the intervals between follow-up reports, incomplete. This probably accounts for the much lower estimates of long-term risk calculated from the cases in SEOPF compared to the KTHS. The difference in long-term projections is quite dramatic: the SEOPF risk factor weights predict virtually no graft losses beyond the first year, while those calculated from the KTHS data predict losses of about 6% per year. Thus, the importance of proper design of follow-up schedules and of restraint in reaching conclusions about results at the outer limits of the time frame of the study is quite evident. The two other prominent differences are a somewhat better graft survival (about 6-8% at one year) in the most favorable transplants, i.e. those involving young, well-matched whites, and a substantially greater detrimental effect of the failure to transfuse preoperatively in the SEOPF data. The last discrepancy may be related to the disparity in the estimates of long-term risk.

Overall, the comparisons described above give substantial encouragement to the application of predictive models especially the one based on the KTHS data as adjunct to decision-making in transplantation.

(b) The Medical Information System of ESRD Program

The Health Care financing Agency has borne the bulk of the costs of dialysis and transplantation as treatments of chronic renal failure since 1972. It is also collecting, through its Medical Information System, voluminous data on both procedures. Until now, this resource has lain essentially unused. The NIAID and the ESRD Program formally undertook to collaborate in the analysis of the data available. For that purpose, computer tapes and their formats were provided to us and the data were installed in the NIH computer.

Because of deficiencies in the data base, prior to analyzing the information on transplantation it was necessary to identify a subset of all putative recipients of kidney grafts for which sufficient data, including a date for the transplantation, were available, and then to deduce for those whose graft had failed, the date of graft failure. Neither task was simple. The selected transplant recipients were quite comparable to the total group in demographic characteristics and therefore represent a satisfactory sample. The available data cover transplants performed in 1977-1979 and followed only until early 1980. Thus, in addition to the flaws intrinsic to the MIS data which resulted from the data acquisition and management system, the problem of short and incomplete follow-up is shared with the SEOPF data. The overall one-year survivals are slightly better than in the KTHS (by about 5%), perhaps reflecting the short followup, and a slightly, but statistically significant difference in graft survival is seen between men and women. These analyses are the first to have been performed on the very large MIS data base and have been made available to the ESRD Program to use as they see fit. Similar demographic and survival analyses have also been performed on the patients on dialysis who have not been transplanted. Comparisons, in terms of mortality, of the two groups cannot be made without adjustments for patient characteristics because of marked dissimilarities. For example, the age distribution of transplant recipients peaks in the fourth decade whereas that for the persons on dialysis increases continuously quite rapidly.

The staff of the MIS have agreed to be trained by us to continue to perform those analyses for which we have written programs. We, in the other hand, will continue to explore new analytical approaches to enhance the utility of the MIS data.

2. Immunosuppression

The retention of a transplanted organ or tissue is virtually totally dependent on effective immunosuppression. Ideally, suppression should be specific and limited to prevention of reaction to the antigens displayed on the graft, leaving the immune system fully capable of dealing with infectious agents or tumor antigens. The induction of antigen specific tolerance can, in principle, be effected by "deleting" (destroying) those lymphocytes bearing binding regions (idiotypes) capable of recognizing the antigens with antiidiotype antibodies (Gordon, Colorado, AI 16825) or with cytotoxic T cells generated by exposure to the idiotype-bearing cells and then cloned and expanded in vitro (Fathman, Stanford, AI 16569; Bach, Minnesota, AIGM 17687). These approaches are, at this time, only in early stages of development. Consequently, the mainstays of immunosuppressive therapy are non-specific including steroids, cytotoxic agents, radiation and antilymphocyte antibodies.

Important developments occurred in the area of non-specific immunosuppression. Total lymphoid irradiation (TLI) as a procedure in which very large doses of radiation are delivered in readily tolerated fractions over several weeks. The target is lymphoid tissue and damage to the bone marrow and lungs is prevented by shielding. Recovery of the immune system from this treatment appears to be characterized by a dominance of suppressive mechanisms. Consequently, there is a greater probability of tolerance of a graft implanted following TLI (Stober, Stanford, AI). The use of TLI to condition human recipients of renal and heart allografts is undergoing initial evaluation at the University of Minnesota.

A more heroic variant, supralethal irradiation and reconstitution with autologous bone marrow to prevent death, is still in the stage of trials on animals (Rapaport, SUNY at Stoneybrook, AI 14453). The rationale is similar. As the lymphoid tissue regenerates from myeloid precursors, it appears to be especially susceptible to the induction of tolerance.

Among the pharmocologic agents, cyclosporin A appears to be in some way selective, giving indications that, if it is used prior to transplantation, a state of graft-specific nonresponsiveness may result permitting discontinuation of the drug. Cyclosporin A is undergoing small-scale trials abroad and in the U.S. in conjunction with organ and with bone-marrow (Thomas, Fred Hutchinson Cancer Research Center, AI 02425) transplantation.

The empiricism that dominates the use of pharmacologic immunosuppressants has long been troubling. The advent of lymphocyte subset-specific monoclonal antibodies has finally provided the opportunity for the analysis of the manner in which the immunosuppressants exert their effect. Although such efforts are only now beginning, some very intriguing observations have already been made. Cyclophosphamide (Cytoxan) at low doses selectively eliminates B cells. At 200 mg per square meter of body surface, it, in addition, selectively reduces the suppressor subset of T cells. At still higher doses selectivity is lost. These dose-dependent effects are reversible. Small maintenance doses of steroids, such as are used in the treatment of systemic lupus erythematosus, have no discernible effects on the immunoregulatory balance as reflected in the lymphocyte subsets. On the other hand, a transient preferential reduction of the helper subset is observed about 6 hours following the intravenous infusion of prednisolone. Azathioprine induces a balanced lymphocytopenia (Schlossman, Sidney Farber Cancer Institute, AI 02650). The effect of antithymocyte globulin is most interesting. Immediately after administration there is a profound lymphopenia. However, recovery from this phase is marked by a predominance of suppressor T cells. (Lee, Medical College of Virginia, AI 12822; Russell, Massachusetts General Hospital, AI 07027). It also appears that the onset of rejection following withdrawal of ATG is preceded by a selective rise in the helper subset.

In a small number of cases, rejection has been treated by the administration of monoclonal anti-T lymphocyte antibody, with careful monitoring of lymphocyte levels. The treatment successfully reversed the rejection (Russell, ibid).

3. Immunogenetics

The major histocompatibility complex (MHC) is a defined chromosomal region within which are encoded proteins which exhibit, in all higher orders, a remarkable polymorphism and which play a major role in the control of immune function. Until the past year or so, virtually all that was known about these proteins and the MHC itself, especially in man, had been learned from studies which employed classical genetic and immunological tools. Amino acid sequences of mouse MHC antigens had been obtained previously, but within the past year extensive sequences of the corresponding human antigens have been determined (Strominger, Harvard, AI 10736). Further work in this area is continuing in a number of laboratories (Silver, Michigan State, AI 16136; Orr, Minnesota, AI/GM 18124; Nathenson, Yeshiva, AI 07289). A modern serological approach to the dissection of the antigenic structure of the human MHC antigens makes use, on the one hand,of the exquisite selectivity of the monoclonal antibodies and, on the other hand,of the wide range of determinants responded to because they are obtained as a result of xenogeneic immunization (Parham, Stanford, AI/CA 17892; Amos, Duke, AI 18399).

The extensive polymorphism of the MHC antigens has consequences which seriously complicate practical applications of their identification to donor-recipient matching in transplantation and in studies on the immunogenetic basis of susceptibility to disease. To date, by far the most extensive work on the serological definition of MHC antigens has been done on the northern and western European (including North American) populations. Efforts to type other ethnic groups with sera obtained from the above Caucasian groups are rendered difficult and the results often of uncertain validity by the existence in the other ethnic groups of antigens totally unrecognized by the Caucasian sera or, conversely, giving unexpected cross reactions with Caucasian antigens. These cross-reactions may result from recognition of broad "public" specificities shared by a variety of antigens (Rodey, Washington University, AI 16574). However, the existence of antigenic differences among the races, presumably the result of evolutionary divergence since the time of separation of the progenitors of the races, offers an excellent opportunity for the study of the evolution of the MHC antigens and of the correlation of their structure with their immunoregulatory functions. As a first step, it is necessary to define serologically the antigens of the various ethnic groups. Their detailed comparison is required next, followed by determination of the structures of antigens which exhibit interesting serologically detected differences. The Branch has issued a request for contract proposals (RFP) to achieve the first objective, improving the characterization of the MHC antigens, for the Black American ethnic group and is negotiating a cooperative venture for similar work in the People's Republic of China. In addition, data collected by the serum Bank in conjunction with its program of distribution of tissue typing trays are being readied for analysis to determine phenotype, genotype and haplotype frequencies among the racial groups in the U.S.

4. Molecular Immunogenetics

The level of activity in this field has increased dramatically in the past year, to the point that new awards for individual research projects and for fellowships, within the purview of GTBB, are made almost solely for work in this area. Whereas in the prior year efforts to determine DNA sequences and organization were focused on genes coding for immunoglobulins, and were amply rewarded, current concern is centered on genes of the MHC (Strominger, ibid; McDevitt, Stanford, AI 18367, AI 07757; Seidman, Harvard, AI 18436, AI 18437). The principal difficulty in this latter endeavor results from the low abundance of RNA coding for MHC proteins, the usual starting point in molecular genetic analysis. This difficulty has now been overcome in the case of the B locus as well as in the case of the accessory B2 microglobulin. Attempts are in progress to isolate RNA coding for DR antigens, or, as portions of their sequences are known, to prepare synthetic polynucleotides to serve as substitute probes for the identification of the genes (Seidman, McDevitt). Although considerable ingenuity and labor are required in the development of detailed procedures, the principles of these steps and of subsequent molecular genetic analyses are well established and straightforward. The two major areas for future development are (1) genetic manipulation by insertion of new genes into cells, and (2) clarification of mechanisms which control activation of genes, effect their rearrangements and result in post-transscriptional modification of mRNA.

5. The Serum Bank

The mission of the Serum bank has been to provide high quality sera and cells for tissue typing to support research in immunogenetics and transplantation immunology. The bank therefore acquired, through purchase and donations, sera recognizing antigens encoded by the A, B, C, and DR loci of the human major histocompatibility complex, and typing cells homozygous at the D locus, evaluated these reagents, and distributed them (the sera in bulk form and on the NIH typing tray) to basic and clinical investigators.

Following the major administrative and functional changes which the Bank underwent in the previous year and in the early part of this year, considerable stability has been regained. The inventory which had been seriously depleted continues to be replenished, although at a slower pace. Thus, 31 liters of sera were acquired, compared to 42 in the previous year, and over 16 liters were distributed, a 25% increase. The Bank is now completely current in terms of offers of sera and their evaluation.

The redesign of the typing tray into a system of Basic, Extended and DR trays is gaining substantial acceptance by the tissue typing community. The redesign was accompanied by a release of the trays for all research applications, not just those connected with clinical transplantation. As a result of both actions the demand for the trays, available only since December 1980, was such that 28,000 were shipped by 6/30/81, compared to about 35,000 of the old style trays shipped in the preceding 12 months. These latter trays remained available until June 1981 and 30,000 were distributed in their final year.

Three additional efforts were initiated through the Serum Bank. The problems and opportunities in the study of histocompatibility antigens of Blacks, described above, were reviewed in a workshop held at Bethesda in December 1980. As a result, a request for contract proposals to screen sera of Black multiparas and compare their reactivity patterns against lymphocytes of Blacks and Caucasians with those of Caucasian sera was issued. The practical objective of this effort is the production of a typing tray specifically applicable to Blacks.

The Serum Bank has also undertaken a program to acquire and evaluate reagents that may be of use in the characterization of polymorphic antigens displayed selectively on various tissues (tissue-specific antigens). This work complements a collaboration with the Immunobiology and Immunochemistry Branch to produce monoclonal antibodies specific for those antigens.

The third major new task of the Serum Bank is a study of the MHC phenotype, genotype and haplotype frequencies for the various races and regions of the U.S. This work is based on the 20,000 or more typings reported every year to the Bank by the users of the typing trays.

IMMUNOBIOLOGY AND IMMUNOCHEMISTRY BRANCH

A. Scope

This Branch is concerned with the biology and chemistry of the immune system and its products. The fundamental studies which it supports on the structure and function of the immune system are directed toward acquiring a complete understanding of immune response mechanisms at their basic cellular and molecular levels as they function in health and disease.

Activities in this broadly-based program area cross traditional disciplinary lines of biology and chemistry and encompass anatomic, physiologic, pharmacologic, and microbial biology and organic, physical, and biological chemistry. Its scope includes studies of the origin, maturation, localization, and function of immunologically active cell populations, and the mechanisms involved in the induction, modulation, regulation, and expression of immune reactivity, as well as physicochemical studies of antigens and their homologous antibodies and the mechanisms and kinetics of antigen-antibody reactions.

Research activites supported within this program area are grouped in the general programs for Immunobiology and Immunochemistry and in a Program of Institute Emphasis (PIE) for Lymphocyte Biology.

B. Awards and Support Levels

Relevant activities in immunobiology and immunochemistry are supported through various mechanisms including contracts, individual post-doctoral fellowships, institutional pre- and post-doctoral training grants, career and career development awards, as well as investigator-initiated research grants.

The following shows the distribution of support by award mechanism for the activities of the Branch during FY 1981.

Immunobiology and Immunochemistry Branch
FY 1981 Awards

Award Mechanism	Number	Amount[1]
Research Grants	269	$30,079,108
Career and Career Development Awards	23	833,603
Subtotal	292	$30,912,711
Fellowship Awards	41	569,107
Training Awards	8	797,944
Subtotal	49	$ 1,367,051
Contracts	4	38,000
Total	345	$32,317,762

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[1] Total costs, indirect costs estimated

The distribution of these awards by discipline is approximately 2/3 for immunobiology and 1/3 for immunochemistry. Approximately 27% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

Support for the Lymphocyte Biology PIE is included in the research grant category. During FY 1981, five program project awards, at a total cost of $3,768,841, were made to support this activity.

C. Program Areas and Highlights

1. Program of Institute Emphasis for Lymphocyte Biology

This Program currently supports five program projects, each of which is a multidisciplinary research effort integrating the technical expertise of cell biologists, geneticists, cellular immunologists and immunochemists and is directed by an acknowledged leader in the field. Their combined studies are designed to expand knowledge of the morphologic and functional heterogeneity of lymphocyte populations and to develop the capability for identification and selection of lymphocyte subpopulations with specific immune reactivity or antigenic composition, for hybridization of such populations and for selective production of specific lymphocyte products. The objective of this Program is to acquire as complete a knowledge as possible of the life history of immunocompetent cells and of the physiologic and external factors that determine their fate and function in vivo and in vitro.

This Program was initiated approximately nine years ago with the award of four program project grants, each directed toward the goal of understanding the biology of the lymphocyte and having different but complementary approaches. Three of the original projects have successfully undergone competitive peer review and have received renewal of support for a five-year period. In addition, support for two new program projects has recently been provided, one each in FY 1978 and FY 1979.

The program project directed by Matthew D. Scharff (AI 10702, Albert Einstein College of Medicine) has focused its approaches on the molecular biology and somatic cell genetics of the immune system to define the genetic and molecular control mechanisms of immunoglobulin synthesis, the structure and synthesis of the antigens of the major histocompatability complex (MHC), and the functions and interactions of T and B lymphocyte populations and macrophages. In many of these studies, continuous cloned cell lines and variants derived from these cell lines are being used to study the role of the cells and their products in the immune response. For example, Dr. Scharff and his associates have devoted considerable time and effort to generate immunologically functional hybrids between mouse myeloma cells and spleen cells at a high frequency and some of the drug marked cells which are now widely used to prepare hybridomas originated in their laboratory. They have provided cell lines and assistance to well over a hundred investigators around the world to establish this technology in their own laboratories.

Studies within the program project have been productive and important contributions have been made. Cultured mouse myeloma cell lines have been used to examine the genetic control of the expression of immunoglobulin genes.

Analysis of antigen-binding variants of these cell lines indicates that the variants arise spontaneously at a very high frequency and may reflect the normal process which regulates the generation of antibody diversity. Primary sequence analysis of these immunoglobulin variants has provided evidence that they have structural changes in the constant region of the heavy chain which cause major changes in the folding of the variable region and produce significant changes in antigen binding. Of interest is Dr. Scharff's recent observations with variants of an immunoglobulin G (IgG)-producing mouse myeloma cell line which synthesizes altered heavy IgG chain. The appearance of a different IgG subclass in some variants probably reflects the activation of previously silent genetic information. Examination of these variants at the protein and nucleic acid levels is expected to provide a new insight into the mechanisms of the heavy chain class switch and of the recombinations involved in immunoglobulin gene expression. Studies of the biochemical properties and primary structure of the products of the H-2 region of the murine MHC are being continued in an effort to relate functional and genetic properties to molecular structure. Discrete amino acid changes in the H-2 glycoproteins of mutant and variant cells have been associated directly with their immunologic specificity.

In order to pursue the mechanisms underlying the complex and coordinated functions of macrophages and to identify the molecular basis for modulation of these functions by products of activated lymphocytes, physiologic and functional studies of cloned macrophage-like cell lines have been initiated. An unusual macrophage-like cell line has been established which can migrate, phagocytize, and respond to the lymphokine, macrophage inhibitory factor (MIF), which affects the migration of normal macrophages. These, as well as other differentiated functions, have not been previously retained in other continuous macrophage cell lines. Because MIF is quantitated only by bioassay, this cell line should prove to be a valuable tool which will facilitate studies on the mechanism of action of MIF, as well as for studies of the functional role of macrophages in infection and immunity. Recent studies have demonstrated that activation of these cloned macrophages induces production of hydrogen peroxide at approximately the same level as activated primary macrophages. This clone and its variants, thus, can be employed as models for study of macrophage oxidative metabolic pathways and the influence of their products on phagocytized microorganisms.

The approach directed by Gerald M. Edelman (AI 11378, Rockefeller University) centers on the analysis of molecules and molecular assemblies involved in the control and recognition of antigenic stimuli at the surface and within the cells of the immune system. The results of his studies have provided several insights into the nature of recognition and control events in lymphocyte function. Because key events such as antigenic stimulation and cell-mediated cytotoxicity rely on the transmission of information from the surface to the interior, detailed knowledge of the mechanisms and cellular constituents involved is important not only for understanding and controlling the immune response but also for understanding the molecular basis of cell differentiation and interaction. In this context, his evidence for the existence of a surface modulating assembly (SMA) which regulates cell surface receptors and mediates transmembrane signalling for functional cellular responses is of special interest. Dr. Edelman and his associates picture the SMA as a three-component system consisting of cell surface receptors that penetrate the cell membrane, submembranous microfilaments which coordinate receptor movement, and microtubules which provide anchorage for the receptors and propagate signals to and from the cell surface.

A long-term objective of this project is to reconstitute a functional SMA. For this effort, a method has been developed to prepare portions of surface membranes from lymphocytes. Using specially prepared beads and antibodies against surface molecules, lymphocytes can be bound to the beads and, after lysis of the cells, patches of membrane remain bound to the solid bead surface. Dr. Edelman and his associates have demonstrated that the inner surface of the membrane is exposed on these patches and that some cytoskeletal elements remain associated with them. Specific antibodies have been prepared against the transmembrane proteins so that their external and internal portions can be distinguished. With these antisera and the surface membrane fragments, it now is possible to analyze the constitutents of the cell surface membrane complex and to define the mechanisms by which antigenic stimuli and other signals are transmitted from outside the cell through the membrane to sites of metabolic activity within the cell cytoplasm. A viral gene protein has been established as a valid probe to dissect the stimulatory sequence leading from the cell membrane to the cytoplasm and the nucleus and to study the enzymes involved in cellular DNA replication. Using this approach, evidence has been obtained which indicates that the microtubules and microfilaments which form the cytoskeleton act as signal regulators in controlling cell growth and division. To further understand the nature of molecular interactions at the cell surface, physicochemical studies of cell surface molecules and of selected external stimuli also are being pursued. A major portion of this effort is concerned with the chemical structure and molecular interactions of the gene products of the murine MHC. Because these molecules are believed to serve as recognition units at the surface of immunocytes, attempts are being made to identify those portions which are involved in interactions with other molecules at or under the cell surface. Of interest is the observation that the heavy chain, as well as the beta-2 microglobulin light chain, contain residues which are phosphorylated. The role of the phosphorylation of these transmembrane molecules in their interaction with other surface, cytoskeletal, and cytoplasmic elements is now being examined to determine its involvement in signal transmission and response controls. To complement this effort and to gain an understanding of these molecular interactions, attempts are being made to define the three-dimensional structure of beta-2 microglobulin using high resolution X-ray crystallography.

Although Dr. Edelman's research is centered on the role of the SMA in the immune response, the implication of his results are broad and suggest that the SMA has a key role in controlling cell movement, growth, interactions and differentiation in general. His recent studies of embryonic neural cells, for example, have led to the detection, isolation and characterization of a cell adhesion molecule (N-CAM) which he found to be involved in the cell-cell interactions required for the orderly formation of cell layers in cultured retinal tissue and for development of nerve bundles. Definition of the role of the SMA in these fundatmental events in nervous tissue development is his next objective. Dr. Edelman also has defined a cell adhesion molecule from embryonic liver (L-CAM) which is involved in hepatocyte aggregation. Specific antibodies against L-CAM not only inhibit liver cell aggregation, but the in vitro formation of histotypic colonies of hepatocytes containing structures similar to bile canaliculi. N-CAM and L-CAM have been shown to be antigenically distinct, and antibodies against each molecule have no effect on cell adhesion involving the other. With the availability of antibodies against L-CAM it should now be possible to examine the relationship of receptor modulation to both liver tissue differentiation and regeneration.

The program project directed by Jonathan W. Uhr (AI 11851, University of Texas Health Science Center at Dallas) is centered on the study of the biochemistry and biology of lymphocyte surface molecules. The structure of immunoglobulins on the membrane of B cell is being studied in order to clarify the molecular interactions that occur after surface immunoglobulins react with specific antigens. The broader purpose of such studies is to obtain an understanding of the molecular mechanisms which underlie signalling of B lymphocytes and control their differentiation. Studies of T cells emphasize the process of immune recognition; efforts are being made to biochemically characterize the T cell receptor and to define the interaction of antigen-pulsed macrophages with T cells. The major concern and principal contributions of Dr. Uhr's project have been in the area of the ontogeny, regulation, and function of B cell surface immunoglobulins. An immunoglobulin D (IgD)-like molecule was found on the surface of circulating murine lymphocytes; ontogenic studies showed that it appeared after immunoglobulin M (IgM) when mice were approximately one week of age. The results of additional studies have led to the acceptance of a model which assigns an important role for IgD in the development and function of B cells. In this model, immature B cells first develop surface IgM, then acquire surface IgD to become "double bearers"; some cells subsequently lose surface IgM to become cells that bear only surface IgD. Dr. Uhr and his associates have demonstrated that cells which bear only surface IgM become tolerized after encounter with antigens. They also have shown that surface IgD acts as a triggering receptor so that cells that bear both IgM and IgD give a primary IgM response after encounter with antigen; cells that bear only surface IgD give an IgG response after encounter with antigen.

In recent studies, he and his associates have employed two types of antigens which activate B cells to produce antibody by different mechanisms to further define the role of surface IgM and IgD. One type can stimulate B cells directly without requiring the help of T cells; the other type apparently depends on the active participation of T cells. From studies using antibodies against B cell surface immunoglobulins, they concluded that T independent antigens require only surface IgM to activate B cells while T dependent responses require both surface IgM and IgD. They have suggested that T independent antigens do not require the participation of surface IgD in B cell activation because they possess an appropriate mitogenic capability which could directly deliver a triggering signal to the cell.

In an effort to relate function to structure, comparative physicochemical studies of membrane and secreted forms of IgM have been continued. Recent data indicate that membrane IgM contains a hydrophobic peptide in the constant region of the carboxy-terminal end of the mu chain which is not present in the secreted form. Although the function of this peptide has not yet been precisely defined, its hydrophobic character suggests that it may have a role in anchoring the molecule in the cell membrane.

Studies of surface receptors on T cells have similarly led to important conceptual advances. Evidence has been obtained to indicate that specific binding of antigens coupled to autologous macrophages by T cells represents the initial event in immune stimulation. The macrophage plays a key role in this stage because T cell receptors do not specifically bind to native exogenous antigens. The results of recent studies suggest that fragments of exogenous antigen, presumably "processed" by the macrophage, and molecular products of the I region of the MHC are the principal molecules involved in lymphocyte recognition of the macrophage surface. They believe that the Ia molecule binds with, and determines the orientation of, antigenic fragments on the surface membrane and, in this manner, appropriately presents the immunodeterminant to the T lymphocyte.

The program project directed by Baruj Benacerraf (AI 14732, Harvard Medical School) is designed to gain understanding of the genetic controls of the immune system and of the regulation of functional expressions of immunocompetent cells. Dr. Benacerraf's outstanding contributions in this field were formally recognized in October, 1980 by award of the Nobel Prize in Medicine. Component activities of his project are concerned with the identification and function of immune response genes, the induction, regulation, and function of helper and suppressor T cells, the identification and function of surface receptors on T and B cells, and the definition of macrophage functions and interactions in the immune response.

Using genetically-determined responder and nonresponder strains of mice, Dr. Benacerraf and his associates have found that specific suppression can be eliminated by administration of antisera specific for I-J gene-controlled determinants; immunopotentiation of nonresponder mice with this antiserum was demonstrated to reflect a reduction of specific suppressor responses. The results of more recent studies have provided evidence that the antigen-specific factor derived from T cells of nonresponder mice may share idiotypic determinants, as well as anti-I-J determinants, within the same molecular complex. These results imply that there are shared variable regions on antibody heavy chains and on T cells which are crucial in the suppression pathway for the same antigen. Related studies on the mechanisms of nonresponsiveness have led to a clear distinction between T cell tolerance and T cell suppression. T cell tolerance exhibited a three-day induction period, had a high inducer cell dose requirement, and was not affected by pretreatment with cyclophosphamide. In contrast, T cell suppression required 7 days for induction, was induced by 1,000-fold fewer cells, and was inhibited by cyclophosphamide pretreatment.

In other studies, macrophages of responder mice were shown to be able to present antigen in an immunogenic form and to play a central role in regulating the balance of activated helper and suppressor T cells. These findings suggested that the predominant suppressor T cell responses in nonresponder mice may be associated with an Ir gene defect at the macrophage level. Of interest in this context is the demonstration that macrophages of neonatal mice do not present antigen efficiently to T cells and that only a small number of these macrophages bear Ia surface molecules. Thus, the ease of tolerance induction and the limited immune response capacity of newborn mice may reflect defective macrophage function but also may reflect a significant mechanism for control of self-reactivity during this developmental period.

Hybridoma technology is being employed to gain further insight into macrophage surface components and their function. The availability of monoclonal antibodies against distinct macrophage surface components will permit more detailed studies of the role of these cells in infections and malignant diseases, as well as in the immune response.

Detailed analysis of the aggregation or capping of surface molecules on B cells during their activation is continuing, with particular emphasis on ionic changes. Recent data indicate that calcium ions are mobilized and released from the cell interior by a transmembrane process when mouse B cell surface immunoglobulins are bound by ligands. The calcium ions changes were not accompanied by a general ionic pertubation within the cell and did not depend on the extracellular ionic environment but was correlated with the cytoskeletal contractile activation which leads to capping of surface immunoglobulins. Mobilization and release of intracellular calcium, thus, may represent an early, physiological, ionic signal for capping.

The program project directed by Carl W. Pierce (AI 15353, Jewish Hospital of St. Louis) has the immunobiology of the MHC as its central theme. The combined efforts of Dr. Pierce and his associates provide a multidisciplinary approach to elucidate fundamental mechanisms of the immune response. Their studies are focused on the biochemistry, genetics, and functions of the MHC, the nature of receptors on immunocompetent cells, and in the diverse regulatory mechanisms of the immune system. Areas currently being examined include the nature of the interactions between histocompatibility gene products and conventional antigens in the generation of effector lymphocytes, the structure of genetically-restricted suppressor factors, the structure and genetic relationships of components of the complement system that are produced by genes of the MHC, the identification and structure of gene products on T cells and macrophages, and the identification and characterization of receptor molecules on the surface of T lymphocytes.

One approach to study the function of these gene products is to construct artificial membrane systems (liposomes) containing isolated H-2 antigens which would stimulate intact cells in various assay systems. At present, liposomes containing H-2 and Ia antigens have been constructed and tested; both types of antigens were demonstrated to be integrally inserted in the liposomal bilayer and to have retained antigenic reactivity in serologic and cytotoxic cell assays. In related studies, a human T cell proliferative assay system has been developed to study gene products of the HLA-D region, the analog of the murine I region. Using this system, preliminary results indicate that human responses are regulated and restricted genetically in much the same way as is the murine immune system.

Chemical differences between Ia molecules in murine macrophages and B cells also are being examined. Tryptic digests of Ia molecules from macrophages and B cells were examined by high pressure liquid chromatography. The beta chains of I-A molecules yielded identical peptide profiles but the alpha chain profiles showed clear differences which are now being studied to define their chemical nature and their possible functional significance. In an effort to obtain a sufficient supply of I region molecules for further study, attempts are being made to clonally propagate murine macrophages and to apply hybridoma technology. T suppressor cells have been successfully hybridized with a thymoma and large quantitites of antigen-specific, Ia bearing, suppressor factor are now being produced for biochemical and immunologic analyses. Preliminary results indicate that the hybridoma-produced suppressor factor has functional activity comparable to that produced by, or extracted from, suppressor T cells. Several suppressor factor producing hybridomas have now been developed, each bearing different H-2 haplotypes. Characterization of this panel of closely related molecules will provide new insights into T cell immunoregulatory molecules which are controlled by the H-2 gene complex.

This Program of Institute Emphasis represents a major financial and philosophic commitment by the IAIDP and the NIAID to definition of the biology of the lymphocyte and the immune system in general. Support for carefully organized efforts by closely interacting groups, led by well-established and highly productive senior scientists, through the program project mechanism has proven to be successful and effective, both scientifically and administratively. The productivity of these select groups has been exceptional and their varied contributions have been, and continue to be, the stimulus for the intense current efforts in this field. In fact, many of the initiatives identified for concentrated development during this decade in the recent report of the NIAID-sponsored Immunology Study Group have been, and are, within the scope of the Lymphocyte Biology Program. In this context, during the past year the Institute considered the future of this Program and decided, with the concurrence of the National Advisory Allergy and Infectious Diseases Council, to continue it in its current form. Accordingly, a Request for Applications was published in March, 1981 announcing a competition for awards in FY 1982. Applications have been received from the two current program projects scheduled for competitive renewal and from an additional investigative group. These applications will be reviewed for scientific merit in October, 1981 and by the Advisory Council in January, 1982.

2. Immunobiology

This program is concerned with the processes leading to immunocyte differentiation, proliferation, and production of biologically-active substances which mediate immune reactions.

Research supported within this program includes studies on the origin, maturation, and localization of immunologically active cell populations, the interactions of lymphocyte subpopulations and their interrelationships with macrophages and other leukocytes, the cellular phenomena of antigen processing, immunologic tolerance and enhancement, and the mechanisms involved in the induction, modulation, and regulation of immune responses. The cellular mechanisms involved in the induction, maintenance, expression and pathophysiology of delayed-type hypersensitive immune reactivity are also included in this category. Also relevant are studies of the lymphokines and other lymphocyte substances which activate macrophages and have pharmacologic effects on lymphocyte and other leukocytic cell functions such as chemotaxis, phagocytosis, blastogenesis, cytotoxicity, and microbial resistance.

A full understanding of the ontogeny of immunocompetent cells is required for selective manipulation of the immune system and for correcting imbalances and deficiencies which compromise host immune defenses. For this reason definition of the origin, differentiative pathways, and functional roles of the various subpopulations of lymphocytes continues to be a focus of considerable investigative effort. Although it is certain that one population of mature lymphocytes (T) is thymus dependent and another population (B) is thymus independent, their progenitors and their mechanisms of functional maturation are still not precisely identified. Robert Auerbach (AI 14607, University of Wisconsin) is assessing the mouse yolk sac as a potential source of immunocompetent cells. Although the yolk sac represents the first source of hematopoietic stem cells and is the first site of erythropoiesis in the embryo, the results of his studies suggest that the yolk sac functions as a primitive effector organ of the embryo rather than a generator of stem cells for subsequent development into lymphoid cells in peripheral tissues. He has found that yolk sac cells can elicit a graft-versus-host reaction and mediate natural cytotoxicity which resembles the natural killer (NK) activity of adult mice. Because NK activity in adults has been identified as pre-T cell function, he suggests that these activities of yolk sac cells may represent a primitive defense mechanism of the embryo which operates until it is replaced by the specific T and B cell responses of more mature individuals.

Studies on the ontogeny of lymphocytes being conducted by Irving Goldschneider (AI 09649 and AI 14743, University of Connecticut Health Center) are centered on the identification and isolation of lymphohemopoietic stem and progenitor cells from murine bone marrow. Using specific anti-lymphocyte sera and the fluorescence-activated cell sorter, he has demonstrated that the Thy-1 antigen, a T cell surface molecule, is present on the least mature members of the B cell series but is absent on mature B cells; for this reason, the Thy-1 antigen can serve as a useful marker of the early stages of B cell development. He also has shown that a subset of bone marrow cells which contain the enzyme terminal deoxynucleotidyl transferase (TdT) is composed almost exclusively of thymocyte progenitors. His recent results indicate that exposure of bone marrow stem cells to thymosin, a putative thymic hormone, can induce TdT expression. The results of related studies conducted by J. John Cohen (AI 11661, University of Colorado) support the view that the thymus, by means of such a hormone, controls production of T cell precursors at the bone marrow level. He believes that T cells acquire their repertoire of cell surface antigen receptors in the bone marrow and that elimination of T cell clones with anti-self reactivity also occurs at this stage.

Although it has been well established that the thymus is the preferred site for normal differentiation and maturation of T lymphocytes, the mechanisms by which the thymic microenvironment influences T cell progenitors are not defined but soluble, perhaps hormonal, factors and cell-cell contact phenomena are most likely involved. David A. Crouse (AI 15819, University of Nebraska) is employing tissue and organ culture technology to evaluate the role of thymic stroma in T cell development. He has found that soluble factors obtained from thymic tissue cultured in diffusion chambers were capable of reconstituting immune responses in neonatally thymectomized mice. Similarly, transplantation of cultured thymic epithelium into athymic mice provided substantial immunologic reconstitution. Ellen Rothenberg (AI 16769, Salk Institute) is attempting to define the sequence of molecular changes that occur in murine T lymphocytes as they mature in the thymus and identify the mechanisms by which specific signals in the thymic environment trigger these changes. As a first step in this effort, she has developed assay systems, based on the appearance of distinct cell surface proteins, to rapidly monitor the responses of medullary and cortical thymocytes to differentiative stimuli.

Studies of T and B cell lineage have been greatly facilitated by the recognition that certain lymphocyte surface molecules are characteristically expressed at specific stages of differentiation and maturation and by the availability of specific antisera to these marker antigens. The products of the Tla region, which is closely associated with the murine MHC, have been demonstrated to govern the expression of surface antigens predominantly on differentiated T cells. Lorraine A. Flaherty (AI 12603, New York State Department of Health) has demonstrated that the Qa antigens, products of a gene within the Tla region, can serve as useful surface markers for identifying immunocompetent lymphocyte subsets. By appropriate breeding experiments, she has identified a series of Qa antigens, each of which appears to define distinct T cell subsets. For example, the Qa-1 and Qa-2 antigens are detectable on thymocytes as well as mature peripheral T cells while the Qa-3 and Qa-4 antigens are found only on mature T cells. The results of recent studies indicate that only the Qa-2 antigen of this series is detectable on bone marrow stem cells. Preliminary evidence also indicates that some Qa antigens are associated with helper and suppressor immunoregulatory functions.

Joan Abbott (AI 13759, Sloan-Kettering Institute for Cancer Research) is studying expression of surface markers to define pathways of murine B cell differentiation. She has found that B cells of newborn mice exhibit surface IgM and the Ia gene product of the MHC but lack surface IgD and complement receptor (CR) while B cells from adult mice exhibit all four markers. When B cells of newborn animals are treated with lipopolysaccharide (LPS) they promptly acquire the IgD and CR markers. She believes the acquisition of IgD and CR reflects an ontogenic progenitor-successor relationship. Also of interest is her finding that B cells from adult mice promptly lose IgD when treated with LPS but retain the CR marker; she suspects that the loss of IgD under these circumstances reflects another differentiation which is an early event in the development of mature antibody-producing plasma cells.

Michael R. Loken (AI 14872, University of Chicago), a recipient of a Research Career Development Award (AI 00348), is developing monoclonal antibodies by hybridoma technology for study of murine B cell differentiation. Three such antibodies have been partially characterized. One identifies both lymphoid and myeloid cells in bone marrow and is believed to recognize a differentiation surface marker which is lost when cells migrate from the marrow. A second antibody identifies all myeloid cells in bone marrow and detects a very early differentiation marker. The third antibody recognizes B cells in all stages, from precursors to mature cells. Recent evidence indicates that the cellular antigen reacting with this antibody is not an immunoglobulin nor is it present in serum. Because this antibody reacts with pre-B cells, he suggests that lymphocytes found in neonatal spleen, liver and bone marrow which lack surface immunoglobulin but react with the antibody represent the earliest precursors of B lineage cells. He is now employing these and related monoclonal antibodies to differentiation antigens to investigate mechanisms regulating normal B cell differentiation and the aberrant differentiation pathways of malignant B cells.

Irving L. Weissman (AI 09072, Stanford University) also has prepared a monoclonal antibody that recognizes B cell and B cell precursors in mice. He has found that the reactive antigenic determinant is not expressed on the hematopoietic stem cell, suggesting that it is expressed later in the differentiation sequence, perhaps at about the same time as the commitment to the

B cell lineage is made. He now is using this antibody in conjunction with the fluorescence activated cell sorter to isolate these cells and study their immunoglobulin biosynthesis and immunoglobulin gene organization. Studies of the molecular and genetic mechanisms involved in the expression of immunoglobulin heavy and light chains during B cell ontogeny should be facilitated by the availability of pre-B hybridoma cell lines which have been prepared recently by John F. Kearney (AI 14782, University of Alabama at Birmingham), a recipient of a Research Career Development Award (AI 00338).

The CBA-N mouse has proven to be a valuable model for study of B cell differentiation. It exhibits an X-linked immune defect in the B lymphocyte population which is believed to result from an arrest of B cell maturation. CBA-N mice lack adult-type IgD-bearing B cells and fail to generate an antibody response to a variety of antigens. Brigette T. Huber (AI 14910, Tufts University) has demonstrated that the Lyb 3 cell surface marker also is absent in these animals. Because her results indicate that the Lyb 3 molecule functions as a triggering receptor on immunocompetent B cells, she believes the absence of this marker on B cells of CBA-N mice is functionally significant. In related studies, John M. Fidler (AI 15226, Scripps Clinic and Research Foundation) has demonstrated that the defect in B cell maturation is not absolute but is age associated. He has found that immune responsiveness in these animals improves with age and suggests that their immune deficit reflects delayed, rather than prematurely terminated, B cell development. He also has demonstrated with appropriate cell transfer experiments that their unresponsiveness does not result from suppressor T cell action.

Another valuable animal model for study of the ontogeny of the immune system has been developed by Henry H. Wortis (AI 15803, Tufts University). He has cross-bred CBA-N mice, which have defective B cell but normal T cell responsiveness, with nude mice, which have defective T cell but normal B responsiveness. The offspring of such matings, unexpectedly, exhibit deficiencies in T and B cell responses. Such doubly deficient mice offer a unique opportunity to explore the genetic mechanisms which affect T and B cell development.

Alexander R. Lawton, III (AI 17996, Vanderbilt University) is studying B lymphocyte development in humans to gain insight into the defects in development and function of B cells which occur in immunodeficiency diseases, autoimmune diseases, and lymphoid malignancies. He has demonstrated that one form of congenital agammaglobulinemia is associated with a defect in differentiation of B lymphocytes from their pre-B cell progenitors while failure of pre-B cell development occurs in a type of hypogammaglobulinemia associated with a tumor of the thymus gland. He now has developed a combined immunofluorescent-autoradiographic technique which permits simultaneous visualization of IgA, IgM, and IgD on human B cells. With this technique, he has found that practically all B cells from healthy neonates exhibit all three markers on their cell surface while no such cells were observed in healthy adults. Patients with various forms of congenital agammaglobulinemia are now being examined by this method in an effort to identify defects in B cell differentiation. He and his associates recently have found that patients with IgA deficiency exhibit the neonatal pattern of surface immunoglobulin markers. This observation suggests that IgA deficient patients have experienced an arrested or abnormal maturation of their B lymphocytes destined for IgA synthesis.

Although the functional distinction of lymphocyte populations is convincingly documented, the purpose and mechanisms of their interactions with each other and with macrophages is still being examined. It is clear that B lymphocytes differentiate to become antibody-secreting plasma cells and that differentiated T lymphocyte subsets can exert helper or suppressor effects on B lymphocytes as well as effector functions in transplantation reactions and in cell-mediated immune reactions to various microbial agents. The cooperative and effector functions of T lymphocytes have been found to correlate well with the presence of the cell surface Ly antigen. It also is known that these functions are controlled and regulated by other cell surface antigens which are products of the genes in the MHC. The regulatory role of MHC products and the functional role of other cell surface antigens in the immune response have been defined most extensively in the mouse although systems analogous to the H-2 component of the murine MHC have been identified in other animals and in man.

On recognition of the regulatory role which the MHC exerts on the immune system, efforts to clarify the responsible mechanisms have intensified. To facilitate research in this area, the program has contractually supported the acquistion and distribution of antisera with specificities against various mouse cell surface antigens. A supply of antiserum against many of the H-2 gene products has been made available for distribution to investigators through a contract awarded to Chella S. David (AI 82564, Mayo Foundation). Similarly, antisera specific for antigens of the I region of the H-2 complex have been prepared previously by Donald C. Shreffler and are available for distribution to investigators. Interest in these reagents remains high; approximately 1300 ml. of these antisera were distributed to 194 investigators during the past year. Of the recipients, approximately one-half were supported by NIH grants; 44 of these investigators were NIAID grantees.

Evidence obtained in several experimental systems has convincingly demonstrated that efficient physiological interactions among macrophages, T cells, and B cells require that these cells share membrane molecules encoded for by the MHC of the species. David H. Katz (AI 13874, Scripps Clinic and Research Foundation) has provided substantial evidence that the genes controlling interactions between T and B lymphocytes are located in the I region of the mouse H-2 complex. He has found that T lymphocytes will not exert effective helper functions for B cells when these cells differ at the relevant I region locus. He believes that self-recognition is the critical mechanism by which cell-cell communication takes place in the immune system. In a related project (AI 13781), he has obtained evidence indicating that the process of differentiation of stem cells and their progeny also may be critically regulated by MHC gene products. Using bone marrow chimeras, he has demonstrated that lymphocyte differentiation may be "adaptive" to the environment in which it takes place. He believes such an "adaptive differentation" results in preferential interactions among cells that have undergone their differentiative processes in the same genotypic environment; "adaptive differentiation" thus may represent a manipulative approach to define genetic and molecular mechanisms responsible for cell-cell interactions.

Genetic restrictions imposed by products of the I region of the H-2 complex on interactions between macrophages and immune T cells are being investigated by Carl W. Pierce (AI 13915, Jewish Hospital of St. Louis).

Using antigen pulsed macrophages, he has found that naive lymphocytes will respond to antigen even if it is presented on a histoincompatible macrophage. However, the secondary immune response is genetically restricted; the immune T cell must be stimulated with antigen-bearing macrophages which are genetically identical to the macrophage that presented the antigen during the primary immunization. He has demonstrated that such genetic restrictions are controlled by products of the I-A subregion of the H-2 complex and believes that these restrictions involve active suppressive phenomena when antigen is presented on an inappropriate macrophage. His recent efforts have been focused on the nature and mechanisms involved in immune suppression by soluble products of lymphocytes and macrophages. A non-specific soluble immune response suppressor (SIRS) produced by stimulated T lymphocytes has been demonstrated to affect antibody responses through macrophages. Available data suggest that SIRS-treated macrophages produce hydrogen peroxide and react with SIRS to generate a macrophage suppressive factor, perhaps the oxidized counterpart of SIRS, which is capable of inhibiting primary and secondary immune responses as well as proliferative responses to T and B cell mitogens presumably by oxidizing cellular components essential for cell metabolism and division.

In a related study, Judith A. Kapp-Pierce (AI 13987, Jewish Hospital of St. Louis), a recipient of a Research Career Development Award (AI 00361), has found that an antigen specific suppressor T cell factor is activated when nonresponder lymphocytes are stimulated by antigen; suppression by this factor is mediated by a molecule encoded by the I-J subregion of the H-2 complex. She also has shown that this suppressor factor does not require participation of macrophages for effective suppression; she suggests that the suppressor factor induces generation of additional antigen-specific suppressor T cells. Furthermore, her recent data indicate that the suppressor factor can directly bind to antigen, thus indirectly interfering the normal pathway of antigen presentation, through macrophages, to helper T cells.

The results of similar studies by Donal B. Murphy (AI 14349, Yale University) suggest that the different functional subpopulations of immunocompetent lymphocytes express different products of the I region on their surface. He has demonstrated that suppressor T cells bear an I region antigen not found on helper T lymphocytes. Of interest is his observation that, although different T cell subpopulations bear distinct I region products, all or many of these products appear to be expressed on the surface of the macrophage so that its interaction with these lymphocytes would not be encumbered by this type of genetic restriction. Recently, he identified a new subset of regulatory T cells which appear to be contrasuppressors, conferring resistance to suppression upon helper T cells. Preliminary evidence indicates that the contrasuppressor system is mediated through a soluble effector molecule produced by these cells.

It is now recognized that the help and suppression provided by T cell subsets is carefully balanced within the responding host. As demonstrated by Richard K. Gershon (AI 10497, Yale University), helper T cells, on stimulating B cells to produce antibody, also become susceptible to regulation through a feedback inhibition mechanism by inducing suppressor T cells. In collaboration with Harvey Cantor (AI 13600, Sidney Farber Cancer Institute), the functional subsets have been identified on the basis of the Ly differentiation antigens present on their surfaces. Thus, helper cells have been shown to be Ly 1+ while suppressor cells are Ly 2, 3+. Dr. Cantor recently has provided convincing evidence that the Ly 1 helper cell can induce an uncommitted Ly 1, 2, 3+ subset to participate in specific suppressor activity. In this context, Dr. Gershon believes from his current studies on the cellular feedback inhibition mechanism that suppressor dysfunction is a cause of at least some autoimmune diseases. Dr. Cantor has demonstrated recently that feedback inhibition is induced by Ly 1+ cells which also express the Qa 1 cell surface molecule and that selective stimulation of Ly 1+ cells which lack the Qa 1 antigen can negate feedback inhibition mechanisms.

Functionally distinct T cell subsets have also been identified in humans using monoclonal antibodies prepared against human thymocytes. Evidence is accumulating that the T4 antigen is present on a mature T cell subset in the peripheral blood population which has helper function; reciprocally, the presence of the T5 cell surface antigen has been correlated with the suppressor population of peripheral T cells. Cornelius P. Terhorst (AI 15066, Sidney Farber Cancer Institute) has provided physicochemical evidence that the T4 and T5 antigens are the human homologs, respectively, of the murine Ly 1 and Ly 2, 3 antigens. He has found that the T4 and T5 antigens can be isolated using antisera against murine Ly antigens and that the isolated human antigens have molecular size and structural features comparable to those of murine T cells.

Transfer of the technology and knowledge of the immunoregulatory system from the murine model to clinical disorders is now occuring in several laboratories. Stuart F. Schlossman (AI 12069, Sidney Farber Cancer Institute) has associated a selective loss of T4 inducer function with anergy and acquired hypogammaglobulinemia in a patient who had normal numbers of B and T cells. John J. Stobo (AI 14014, University of California at San Francisco) has determined that some patients with common variable immunodeficiency have deficient T helper cells and has found deficient macrophage populations in other patients. In another approach, Anthony J. Strelkauskas (AI 16651, Medical University of South Carolina) has been investigating the presence of auto-antibodies in clinical immunoregulatory disorders. He has found that some patients with juvenile rheumatoid arthritis exhibit auto-antibodies against suppressor T cells while some individuals with agammaglobulinemia exhibit anti-helper T cell reactivity. Recently, he has demonstrated auto-antibodies against suppressor T cells in aged individuals exhibiting immunoregulatory deficiences.

Although research on functional subsets of T lymphocytes is proceeding at an accelerated pace, it is recognized that this work is technically limited by the need to separate the subsets in adequate numbers for study. This difficulty may be obviated by the recent work of Frank W. Fitch (AI 04197, University of Chicago) who has developed a method to isolate and culture cloned lines of reactive T cells. Two types of T cell clones have been derived. One is cytolytic and expresses Ly 2 alloantigens; the other is non-cytolytic, expresses Ly 1 antigen, and exhibits helper functions. The latter, in mixed culture, can promote proliferation of cytotoxic cell clones and, in isolated culture, generates a soluble factor which can activate cytotoxic cell clones. Another approach is that being explored by Robert T. DeMars (AI 15486, University of Wisconsin) who is attempting to produce T cell hybrids by fusing immune mouse T cells with mouse T lymphoma cells.

Preliminary results are encouraging; the hybrids express the Thy-1 antigen of normal lymphocytes and produce soluble factors which suppress cytotoxic cell activation. The development and improvement of technology for T cell cloning can be expected to markedly facilitate studies of cell-cell interactions and of immunoregulatory mechanisms in the future.

To assist current research efforts, the IAIDP has employed the contract mechanism to provide useful research reagents. The acquisition and distribution of Ly antisera and the preparation of antisera against newly-recognized Ly antigens and other immunologically-relevant cell surface molecules are being supported by a contract with Edward A. Boyse (AI 82541, Sloan-Kettering Institute for Cancer Research). During the past year, this contract provided antisera to 44 investigators, approximately one-half of whom were NIAID grantees. The recently-awarded contract to Anne W. Hamburger (AI 02655, American Type Culture Collection) for the acquisition, characterization, storage and distribution of hybridoma cell lines also is intended to support and facilitate investigator-initiated research studies of the immune system. During the first year of operation, approximately 50 hybridoma cell lines have been acquired and are being characterized and propagated for subsequent storage and distribution. Approximately 10 such lines are near final characterization and should be available for distribution by October, 1981.

3. Immunochemistry

Research grouped in this program category focuses on the defined molecular components of the immune system with particular emphasis on elucidating the molecular basis of the regulation of the immune response utilizing chemical and physicochemical techniques for study of immunologic reactants and reactions. A major aim of studies supported by this program is to elucidate the structure of antibodies with emphasis on their antigen binding, specificity, and other biological functions. A second objective of this program is to elucidate the critical aspects of antigen structure that determine immunogenicity of the antigen.

The results of structural studies have established that an immunoglobulin basically consists of two identical light (L) and two identical heavy (H) chains, linked by disulfide bridges to form a two-fold symmetrical molecule, each side of which consists of an L and an H chain. Two classes of L chains (kappa and lambda) and five H chains (gamma, alpha, mu, epsilon and delta), which determine the immunoglobulin class (IgG, IgA, IgM, IgE, and IgD, respectively) have been recognized. Limited proteolysis of a typical IgG molecule results in three fragments of nearly equal size, 2 Fab fragments and an Fc piece. It is now clear that Fab components contain the antigen binding (combining) site and that the Fc fragment plays an important role in complement fixation. Amino acid sequence studies of immunoglobulins have demonstrated the existence of three domains with constant sequences on the heavy chain (C_H) and one domain of variable sequence (V_H); each light chain consists of two domains, a constant region (C_L) and a variable domain (V_L). The close association of the V_L and V_H domains in the Fab fragment, containing the combining site, produces a continuous hypervariable surface which can be readily altered by amino acid substitution, insertions or deletions and provides an extremely large number of structural combinations which are believed to provide structural diversity and to confer immunologic specificity.

Amino acid sequence analysis and structural studies provide one approach to understanding immunoglobin diversity and function. Modern technology and instrumentation have had a profound impact in this area. Leroy W. Hood (AI 14059, California Institute of Technology) has developed a new sequenator employing a solid-phase support and gas-phase Edman chemistry. This instrument has several striking advantages over older models. The gas-phase chemistry speeds up the reactions by a factor of 2 to 3; the reaction chamber is very small and, accordingly, uses one-twentieth of the solvents and reagents currently required. Dr. Hood believes that this instrument should permit analysis of one picomole of polypeptide, an amount which can readily be derived from cell extracts by a high-resolution two-dimensional gel separation system.

A key element in the regulation of the immune response concerns unique antigenic determinants (idiotypes) associated with variable regions of antibodies, receptors and antigen-binding factors. These idiotypic markers constitute a target for the regulatory action of idiotype specific antibodies, T lymphocytes and factors. Significant progress has been made recently in elucidating aspects of the regulatory process which involve idiotype-anti-idiotype interactions. Alfred Nisonoff (AI 12895, Brandeis University) has demonstrated that it is possible to suppress the major idiotypic component of the humoral response to the azophenylarsonate (Ar) hapten with little effect on the total production of anti-Ar antibodies. He found that injecting mice with rabbit anti-idiotypic antibody prior to immunization with the protein-hapten conjugate (KLH-Ar) results in the induction of a large population of idiotype specific suppressor T cells with anti-idiotype receptors. Anti-idiotype treatment also was shown to suppress delayed type hypersensitivity by the action of suppressor T cells. The results of related studies demonstrated that idiotype bearing factors are derived from T cells which themselves have idiotypic receptors. He has concluded from his studies that the regulation of idiotype expression is not limited to suppression by the action of T cells or anti-idiotype antibodies. In some situations, anti-idiotypic helper T cells play an important role in promoting the expression of an idiotype; idiotype expression can also be favored in some instances by administration of anti-idiotype antibody, certain classes of which can, under appropriate conditions, prime B cells expressing that idiotype. Eli E. Sercarz (AI 11183, University of California at Los Angeles) has demonstrated that the murine secondary antibody response to hen egg white lysozyme (HEL) consists predominantly of a single idiotype (IdX-HEL). Of interest is his finding that antibody-producing B cells obtained within the first week after immunization with HEL did not secrete the IdX-HEL antibody while 80 to 100% of the B cells obtained two weeks and later after immunization exhibited IdX-HEL. He believes that an idiotype specific T helper cell is critical to development of this response; it may play a crucial role in the selective stimulation of IdX-HEL bearing B cells. In another experimental model, Joel W. Goodman (AI 05064, University of California at San Francisco) has defined the major idiotype of anti-azobenzearsonate antibodies. He has identified this idiotype on ABA-binding T cells and has isolated an idiotype-expressing molecule from these cells.

It is evident that further work is needed in the area of cell regulation by idiotypes. The controlled manipulation of anti-idiotype regulatory systems may have important potential for the regulation of both the normal human immune responses and responses in immunologic disorders. It also is possible that regulation of anti-idiotypic immunity may offer a strategy for control of tumors of the immune system, such as the myelomas and lymphomas.

Progress has been made using model membranes, liposomes, to analyze the antigenicity of membrane localized antigens. The membrane properties which are most likely to influence the immunogenicity of a membrane-localized antigen include the accessibility, amount and distribution of the determinant, the presence and proximity of other antigens, and the surrounding physicochemical environment. These parameters can be readily manipulated in the preparation of liposomes and, accordingly, Stephen C. Kinsky (AI 15976, National Jewish Hospital and Research Center, Denver) has performed in-depth analyses using such model membranes. The liposomes employed in Dr. Kinsky's studies were rendered immunogenic by incorporation of synthetic amino-substituted derivatives of phosphatidylethanolamine (PE). To date, 16 derivatives have been synthesized that differ in either the nature of the hapten substituent or the spacer by which the hapten is attached to the amino function of PE. By varying the structure of the derivative, the amount incorporated, and the phospholipid and sterol composition of the liposomes, major factors that influence the response to a membrane-localized antigen have been analyzed. All the liposomes examined to date produced a thymus-independent humoral response with only one exception. The magnitude of the response was found to be dependent on both liposomal dose and determinant density; factors critical to development of a response included the phospholipid composition of the membrane and the presence of amphipathic derivatives on the antigen as well as the maintenance of an effective spatial separation between antigenic determinants.

The fundamental concept that T cells and B cells may recognize and respond to different determinants of an antigen molecule, initially proposed in the late 1960's, has received sufficient experimental support in recent years to place it among immunologic dogma. The model emanated from studies using conventional haptenated proteins; it was shown that recognition of at least two determinants, presumably one by each of two collaborating cell types, was required for an antibody response. Firm evidence for this concept has been derived from studies using relatively simple, structurally defined, antigens. Using glucagon, a polypeptide of 29 amino acids, Joel W. Goodman (AI 05664, University of California at San Francisco) demonstrated that this compound is immunogenic in guinea pigs and could be dissected by tryptic digestion along functional lines into an amino-terminal portion which housed the major haptenic determinant against which the antibody-forming B cell response was directed and a carboxy-terminal region which carried a determinant that induced T cell responses. The use of such a defined or synthetic antigen to clarify further the types of determinants which activate specific components of the immune system could be useful in the design of immunotherapeutic approaches.

MICROBIOLOGY AND INFECTIOUS DISEASES PROGRAM

NIAID

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MICROBIOLOGY AND INFECTIOUS DISEASES PROGRAM

NIAID

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MICROBIOLOGY AND INFECTIOUS DISEASES PROGRAM

Annual Report, 1980-81

Director's Report

Introduction

In the fall of 1980, the DHHS publication Promoting Health/Preventing Disease: Objectives for the Nation noted that in 1976 deaths attributable to infectious diseases as a group were surpassed only by cardiovascular diseases, cancers, and stroke. Collectively, infectious diseases ranked fourth as a cause of death. Mortality, however, is an inadequate measure of the impact of infections on the health of Americans. Morbidity, as reflected by disability and non-effectiveness, is a much better one. To examine the impact of infections and infectious diseases, staff reviewed the code used by the National Center for Health Statistics for its 1976 combined statistics and refined the listing to summarize morbidity attributable to those illnesses for which MIDP is responsible. As shown in the following table, although infections accounted for only 10% of all hospital days, they were responsible for 68% of all acute bed days. When acute and chronic disability were combined, infections accounted for 70% of days lost from school and work.

Impact Category	Number x 100 Total	 Infections	Per Cent Infections
Deaths	1,909	92	4.8
Hospitalization			
Discharges (cases)	38,639	4,133	10.7
Days (days)	282,766	29,244	10.3
Doctor's Office Visits	588,300	122,663	20.9
Chronic Disability			
Reduced activity (days)	2,394,618	89,765	3.8
School and work loss (days)	208,622	12,198	5.9
Prevalence (cases)	116,962	10,757	9.2
Incidence (cases/year)	15,890	2,638	16.6
Acute Disability			
Reduced activity (days)	2,014,813	1,220,379	60.6
School and work loss (days)	534,831	343,485	64.2
Bed days (days)	931,601	635,180	68.2
Incidence (cases/year)	461,377	322,064	69.8

The cost of all of these illnesses has not been accounted, but Dr. J. W. Curran of CDC provided estimates for one of them during the year, pelvic inflammatory disease (PID). Due principally to Neisseria gonorrhoeae and Chlamydia trachomatis, this consequence of sexually transmitted infections ocurred at an annual rate of 850,000, requiring 2,500,000 physician visits, 212,000 hospital admissions and 115,000 surgical procedures. Ectopic pregnancies in the U.S. tripled from 1967 to 1977. Direct and indirect costs of PID and PID-associated ectopic pregnancy exceeded $1.25 billion in 1979. The mission of the NIAID is to find better ways to prevent and to treat these and all other infections. The accomplishments of the four branches of the Microbiology and Infectious Diseases Program (MIDP) are summarized in the following sections; activities in International Health are reviewed at the end of this section. The major focus of this year's scientific summary is vaccine development.

Administrative Summary

Clinical and Epidemiological Studies Branch: In the fall of 1980, Dr. George Curlin, Chief of the Epidemiology and Biometry Branch and Enteric Diseases Program Officer, transferred to the Agency for International Development. The Epidemiology and Biometry Branch became a section of the Clinical Studies Branch, the name of the Branch being changed to reflect this combination. Dr. Richard Kaslow is Head of the EB Section. Dr. Alfred Saah, an Epidemiology Associate assigned to NIAID, is now working full-time with the staff following completion of his MPH at Johns Hopkins. Dr. Robert Edelman, Chief of CESB, also became Enteric Diseases Program Officer and Executive Secretary of the U.S.-Japan Cholera Panel. He is assisted in these duties by Ms. Jacqueline Smith.

Other Personnel Changes: Dr. Maureen Myers succeeded Dr. June Dunnick as Antiviral Substances Program Officer in the Development and Applications Branch. Dr. Harley Sheffield transferred from IAIDP to MIDP to succeed Dr. Kenneth Phifer, who retired from the PHS, as Parasitology Program Officer in the Molecular Microbiology and Parasitology Branch.

STD Status Report: During the year, the full report of the NIAID Study Group on Sexually Transmitted Diseases was published. We are indebted to Dr. King Holmes, Chairman of the Study Group, and to the 65 members of the nine panels for this excellent overview of the STD problem and for their assessment of research needs.

Consensus Conference: A Consensus Development Conference sponsored by NINCDS and co-sponsored by NIAID, NIADDKD, NIEHS, NICHD, and DRR was held on March 2-4, 1981, to address issues on the diagnostic criteria and treatment of Reye's Syndrome. Dr. John LaMontagne, Influenza Program Officer, DAB, serves as Chairman of the trans-NIH Reye's Syndrome Working Group.

Workshops: The following workshops were organized by MIDP staff:

January 9	Potential of Human Immune Globulin for the Prevention and Treatment of Bacterial Diseases
February 2-3	Concept Review of Genital Herpes Vaccines
May 11-12	Maternal Genitourinary Infections and the Outcome of Pregnancy
May 12-13	Antivirals and Genital Herpes

June 18-19 Serodiagnosis of Systemic Mycoses

June 27-29 Bacterial Virulence and Pathogenesis

Commercialization of Biomedical Research: It is appropriate to note that during the year, two technologies spawned in large part by NIAID-supported investigators - recombinant DNA technology for genetic engineering and hybridoma technology for the production of monoclonal antibodies - presented universities with both the opportunities and risks of new commercial possibilities as scientists and administrators explored mechanisms for collaboration between academic institutions and industrial corporations. Since technology transfer is a desirable objective, the mechanisms devised to foster application of these two technologies while at the same time seeking to prevent the profit motive from distorting scientific research will merit careful attention.

Vaccine Consultant: For two weeks during March, at the time of his visit to the U.S. to participate in a conference on influenza, MIDP was privileged to enjoy a return visit by Sir Charles Stuart-Harris of the University of Sheffield. He consulted with staff regarding vaccine research and development, with particular attention to the roles of the Vaccine Evaluation Centers (Baylor, Marshall, Michigan, Rochester, Vanderbilt, Vermont) and the Center for Vaccine Development (Maryland). Suggestions made at the time and in his subsequent written report have been most helpful.

Accelerated Vaccine Development: In the fall of 1980, the Secretary of the Department of Health and Human Services accepted the recommendation of the HHS Steering Committee for the Development of a Health Research Strategy that an NIAID proposal for the "Accelerated Development of New Vaccines" be added as one of four new initiatives to 11 prior initiatives identified in December of 1979, the 15 initiatives to constitute the Department's proposed initiatives for Fiscal 1981. All initiatives represent research programs that derive special force from participation of two or more agencies, with the initiative for Accelerated Development of New Vaccines involving the Centers for Disease Control, Bureau of Biologics/Food and Drug Administration, and four other Institutes of the National Institutes of Health as co-sponsors in addition to NIAID as the sponsoring agency. The purpose of this new vaccine development initiative is to develop within DHHS a clearly-identified, coordinated, and recognized approach to the further conquest of vaccine-preventable disease. The incentive for expanded efforts lies in new knowledge and processes emerging from recombinant DNA and hybridoma technology and in the better understanding of the workings of the immune system. The goal of the initiative is to expedite the availability of needed vaccines by selecting a few candidate vaccines for extra effort with additional funding. Studies are now in various stages of progress on 53 vaccine antigens for 28 different bacterial, viral, and parasitic diseases, the majority of these studies being dependent on investigator-initiated projects. With the assistance of existing advisory committees and "state-of-the-art" reviews by workshops, and in coordination with the newly-appointed PHS Interagency Group to Monitor Vaccine Development, Production, and Usage, it is proposed that a few candidate vaccines could be brought into use several years earlier than might otherwise be the case. To aid in this process, the proposed initiative called for a review of potential vaccine-preventable diseases from the standpoint of socio-economic and medical needs and for an assessment of the cost/benefits of vaccines for each of these diseases. The Institute of Medicine has been asked to undertake this review and evaluation so as to assist NIAID in setting priorities for development.

Scientific Summary

The Surgeon General's Report on Health Promotion and Disease Prevention (1979) entitled HEALTHY PEOPLE states that "Prevention is an idea whose time has come," and cites past success in the struggle against infectious diseases. A large part of this success is attributable to immunization, one of the most effective preventive measures employed on behalf of the public health. In keeping with the initiative for the accelerated development of new vaccines, some recent studies related to vaccines constitute the major body of this summary.

Bacterial Adherence: A phenomenon related to new approaches to immunization is that of bacterial adherence to mucosal surfaces, an aspect of bacterial pathogenicity that has received increasing attention in recent years. A discussion of this subject at the Institute-sponsored workshop in July on bacterial virulence and pathogenesis was lead by Edwin H. Beachey, M.D. of the University of Tennessee School of Medicine. In his review of the subject at the fall meeting of the Infectious Diseases Society of America, and in a recently-published book edited by him, Dr. Beachey noted that a number of investigators have now shown that, in general, the ability of certain bacteria to adhere to epithelial cells *in vitro* parallels their ability to produce infections *in vivo*. The premise of these studies is that the adherence of bacteria to tissue surfaces involves specific interactions between molecular ligands, referred to as adhesins, on the surface of the bacteria with specific structures, referred to as receptors, on the tissue surface. It is anticipated that the isolation and identification of the adhesin and receptor materials from each of these interacting surfaces will provide new approaches to the prevention of bacterial infections. In the case of the chemical component or attachment organelle that serves as the specific bacterial adhesin, vaccination using the purified adhesin as an antigen may produce immune responses resulting in the local mucosal secretion of anti-adhesin antibody. Reaction to antibody with adhesin, it is assumed, would block bacterial adherence. This is the rationale for the development of pili vaccines for *E. coli* and gonococci, since pili are the fimbriated structures that attach these organisms to mucosal surfaces.

Studies of a non-adherent mutant of an enterotoxigenic *E. coli*, a strain lacking colonization factor, showed that it failed to produce diarrhea in human volunteers even though it retained its ability to produce enterotoxin. Thus, bacteria must adhere to mucosal surfaces not only to survive but also to enable them to deliver their toxic material to the host cell membranes. Theoretically, an inactivated vaccine that would stimulate the production of local antibody to colonization factor (pili) would not only block colonization but also prevent elaboration of toxin, if any. An interesting variation of this approach, recently tried with another toxigenic organism, *Vibrio cholerae*, is to select a live mutant that will attach - and presumably immunize - but lacks the ability to produce toxin.

Other studies of *E. coli* infections in animals and man support the argument that adherence of microorganisms to mucosal surfaces is an essential first step in the pathogenesis of bacterial infection. Studies of diarrhea in piglets induced by K88 strains of *E. coli* have shown that the intestinal epithelial cells of susceptible piglets bind large numbers of *E. coli* while epithelial cells of resistant animals bind few or none. Crossbreeding experiments have shown that the binding capacity of intestinal brush borders and the susceptibility to diarrhea are autosomal dominant traits, with the receptor for K88 strains of *E. coli* missing in resistant piglets. This is further evidence that the bacteria interact with host cells by specific molecules of recognition. Pertinent to immunization in the demonstration by Dr. C. D. Brinton and associates that the vaccination of pregnant gilts with purified E. coli pili confers passive protection to neonatal pigs against diarrheal disease caused by enterotoxigenic E. coli strains that possess the same pili. Early trials of E. coli vaccines in human volunteers have shown only limited protection to date.

Perhaps the most common human infection caused by E. coli involves the urinary tract, particularly in females. Investigators have now shown that E. coli adhere in much greater numbers to the periurethral cells of females who have recurrent urinary tract infections than to epithelial cells from those who have not had such infections. It has been shown that pyelonephritogenic strains of E. coli bind to specific glycolipid structures on the surface of uroepithelial as well as red blood cells. The quantity of these structures - composed of hexosylceramides - appears to vary genetically from individual to individual, and it has been suggested that the relative susceptibility to E. coli-induced pyelonephritis is related to density of such receptor structures on host epithelial cells. An alternative to the vaccine approach, then, might be the application of synthetic analogs of receptor materials as competititve inhibitors of the adherence of bacteria. Such an approach would be easiest to apply to pharyngeal infections such as those caused by streptococci. Dr. Beachey now believes that the adhesin for Group A streptococci is a complex of leipoteichoic acid (LTA) and LTA-binding protein which bonds to an albumin-like host cell receptor. With regard to group B streptococci, in vitro studies of Dr. Barbara Sanford have shown that these bacteria bind to tissue culture cells infected with influenza virus, but not to uninfected cells. The capacity of viruses to predispose to secondary bacterial infections by altering adhesin-receptor relationships needs further exploration.

Among the anti-adhesin vaccines, one of great current interest is that prepared from gonococcal pili. Two groups of investigators have now initiated early human trials of gonococcal pili vaccines, having demonstrated immune responses to the purified antigens. Prevention of adhesion alone may not suffice, however, and it may be necessary to combine pili antigens with outer membrane antigens that have been shown to promote opsonization. Further, gonococcal "toxins" may be involved. While assessing the relative virulence of piliated and non-piliated gonococci in organ cultures of human fallopian tubes, Dr. Zell McGee and associates at Vanderbilt observed that piliated gonococci were more destructive than non-piliated ones. The damage to the ciliated mucosal cells was mediated by a subcellular toxic factor(s) identified as gonococcal lipopolysaccharide (LPS). Here, then, is another example of attachment facilitating delivery of toxin to target areas. To determine how many pili types must be included in a vaccine, and whether or not they must be combined with membrane antigens without LPS, will require extensive study. The recent appearance of strains of gonococci resistant to both penicillin and spectinomycin has given added impetus to the need to accelerate such research.

Promising New Vaccines: A number of new bacterial and viral vaccines developed in recent years are making their way through various levels of testing. Some will soon be ready for application in selected populations; others are ready for expanded clinical trials. The most promising, not all of which are being sponsored by NIAID, are the following:

Rabies: Human diploid cell strain rabies vaccine, inactivated, produced by a French firm, was licensed in the U.S. for distribution through public health agencies. It is much more effective than duck embryo vaccine, requiring only five doses for post-exposure prophylaxis rather than 21. A license application from a U.S. producer is pending. Studies are now in progress to assess the extent of antigenic variation among street rabies viruses detected by analysis with a panel of monoclonal antibodies. It may be necessary to include more than one strain in the vaccine, at least in certain parts of the world.

Hepatitis B: The inactivated surface antigen vaccine developed by Merck, Sharp and Dohme has been shown to be highly efficacious in male homosexuals. U.S. licensure is imminent. A similar vaccine developed at NIAID has been shown to be safe and highly antigenic. A trial in China where maternal HBs Ag carrier rates are high is planned to test the efficacy of this vaccine in preventing neonatal transmission. A French-made vaccine has been effective in Senegalese children, reducing the incidence of the HBsAg carrier state by 85% in susceptible children at the twelfth month of follow-up. Anti-HBs antibody of maternal origin did not interfere with active immunization, so vaccine administration will begin soon after birth. If, as strongly suggested by epidemiological data, chronic hepatitis B virus infection is a prelude to hepatic cell carcinoma (hepatoma), prevention of infection early in life should prevent virus-related hepatoma later in life. Liver cancer (presumed primary) is the third most important cancer as a cause of death in China, accounting for 15% of all cancer deaths.

Meningococcal Meningitis: A bivalent meningococcal vaccine containing groups A and C polysaccharides has been licensed for several years, particularly for use in military recruits. Other meningococcal groups, notably V, Y, and W135, continue to cause meningitits both in the U.S. and abroad. It is anticipated that tetravalent vaccine containing A, C, Y, and W135 polysaccharides will soon be licensed and marketed by Connaught and Merck. Unfortunately, group B polysaccharide alone is a poor antigen, so it has been combined with a group B protein by scientists at the Bureau of Biologics to improve its immunogenicity. Should trials now underway demonstrate its effectiveness, the group B complex may be added to formulate a pentavalent vaccine.

Hemophilus Meningitis: A vaccine made by combining *Hemophilus influenzae* type B polyribose phosphate with a small amount (approximately 1/6 of the childhood dose) of inactivated pertussis cells has been made by Lederle Laboratories. Preliminary studies indicate that this complex is antigenic in children under six months of age when given in three doses. It is assumed that protein conjugation converts the polysaccharide from a T-cell independent to a T-cell dependent immunogen. MIDP wishes to collaborate with the manufacturer in conducting an efficacy trial in Alaskan Eskimo populations with a high incidence of meningitis. In the U.S., there are approximately 15,000 cases each year, and 10 to 35% of the strains are now resistant to ampicillin.

Chickenpox: An attenuated varicella vaccine developed by Japanese scientists has been tested in over 2,000 normal children in Japan. The vaccine is being produced in the U.S. by Merck. Clinical studies supported by NIAID are in progress to determine the efficacy of this live, attenuated vaccine in protecting leukemic children at high risk of developing serious disease when exposed to siblings or other children with chickenpox. Since herpes zoster (shingles) is a late consequence of natural chickenpox, it can be anticipated that use of such a vaccine - if shown to be effective - in normal children will soon be the subject of lively debate.

Typhoid Fever: A live, oral, attenuated typhoid vaccine developed by Dr. R. Germanier of the Swiss Serum and Vaccine Institute promises to be much more effective and much less reactogenic than currently-used inactivated typhoid vaccines. The vaccine is prepared from a *Salmonella typhi* mutant (Ty2la) that lacks the enzyme UDP-4-galactose-epimerase and is capable of only incomplete synthesis of cell-wall lipopolysaccharide. The protective efficacy of Ty2la in human volunteers was first demonstrated by Dr. Richard Hornick and associates at the University of Maryland. In addition to protecting against disease, the vaccine stimulates local gut immunity that decreases the excretion of virulent *S. typhi*. A placebo-controlled trial in 32,000 Egyptian school children then showed the vaccine to be highly protective. Investigators at Maryland are now seeking approaches to oral administration of the vaccine to volunteers in the hope of finding one that will not require the concommitant administration of bicarbonate. Pilot studies in U.S. adults and Chilean school children have shown that vaccine administered in enteric-coated capsules is immunogenic and not associated with any adverse effects. Dr. Myron Levine has planned a field trial in Chile this fall.

Rocky Mountain Spotted Fever: It had been hoped that a new inactivated whole cell vaccine developed by the U.S. Army could be added to this list. Despite its promise in guinea pigs and monkeys, and its immunogenicity in humans, two doses of the vaccine failed to provide adequate protection in volunteers challenged with virulent *Rickettsia rickettsii*.

Antiviral Drugs: The search for synthetic compounds that specifically inhibit enzymes encoded by viruses is accelerating. Acyclovir, an anti-herpes drug that is dependent for its action on phosphorylation by a virus-specified thymidine kinase, is being compared to adenine arabinoside (ara-A) and adenine arabinoside monophosphate (ara-AMP). Ara-A has been shown to be efficacious against herpes encephalitis. During the year, a study was published showning its efficacy against neonatal herpes, a serious infection that is receiving increased attention because of its association with maternal herpes genitalis. Ara-A also seems to decrease the severity of herpes zoster in the immuno-compromised host.

Toxic Shock Syndrome: During 1979 and 1980, there was a marked increase in the U.S. in the number of cases of an illness manifest by fever, drop in blood pressure, vomiting, diarrhea, and a desquamating rash. Ninety per cent of the cases involved menstruating women using tampons. About 10% of the cases were fatal. Microbiologic studies associated *Staphylococcus aureus*, resistant to penicillin, with the illnesses, the clinical manifestations of which suggested the presence of a toxin produced by the bacterium. In mid-year, two groups of NIAID-supported investigators reported that strains of *S. aureus* isolated from 94% to 100% of patients with toxic shock syndrome produced an enterotoxin-like protein. Dr. M.S. Bergdoll and associates at the University of Wisconsin (*Lancet*, May) tentatively identified the toxin as enterotoxin F. Dr. P.F. Schlievert *et al* at UCLA (*J. Infect. Dis.*, April, published June) called the toxin exotoxin C. Enterotoxin F and exotoxin C are said to be identical. Only 5% to 16% of *S. aureus* strains from other sources produced a toxin. These observations will facilitate additional studies of the epidemiology and pathogenesis of this puzzling syndrome.

International Health

International health research programs are administered by Dr. Earl Beck, Special Assistant to the Director, MIDP, in conjunction with individual program officers and coordinators.

International Collaboration in Infectious Diseases Research (ICIDR) is a program initiative that was started in September, 1979. The program is divided into Part A, International Program Project Grants, and Part B, International Exploratory/Developmental Research Grants, to provide increased programmatic flexibility and to insure maximum opportunity for investigator participation and collaboration. The research emphasis of the ICIDR program is infectious diseases and the immunology of these diseases, and includes malaria, schistosomiasis, filariasis, trypanosomiasis, leishmaniasis, and leprosy.

Part A, International Program Project Grants, embraces broadly based multidisciplinary research programs with a well-defined central focus or objective, and with the major portion of the research (80%) being conducted overseas with an acceptable foreign affiliate in a developing country. There are currently 5 program projects; 4 are completing their 02 year, and one has just completed their 01 year.

Part B, International Exploratory/Developmental Research Grants, is designed to encourage the individual investigator to develop a biomedical research program with an overseas affiliate, with the major portion of the work performed in the host country. These are planning type grants that should lead to the development of a large program project (P01) or a regular research grant (R01), or a foreign grant (F). Currently, there are 5 exploratory/developmental grants completing their 02 year.

The ICIDR program continues to generate interest throughout the scientific community. Readvertisement and continuation of the program will depend upon Institute policy and the availability of funds.

The 10 institutions and the U.S. principal investigators participating in the ICIDR Program and their respective areas of research interest are:

Part A (P01) - Program Project Grants

1. Cornell University Medical College, New York City, with Drs. Thomas Jones and Warren Johnson as Co-Principal Investigators. Collaboration is with the Federal University of Bahia and with the University of Brasilia in Brazil on cutaneous, mucocutaneous, and visceral leishmaniasis and trypanosomiasis. The investigators report that administratively everything is proceeding smoothly and effectively. A one-day symposium on research activities of Brazilian and American investigators on the project was held at the University of Bahia in February, 1981. The exchange of information among Brazilian researchers has proved to be an important reward of the project and of great benefit to research oriented young investigators in Brazil. In addition, the opportunity to present their research at national and international meetings has stimulated or renewed interest in research at the University of Bahia. The peripheral effects of the collaborative activity have included use of the basic program project model for requests of other investigators for funding from Brazilian research funding sources, such

as CNpQ, the WHO, and the Rockefeller Foundation. These "ripple effects" of the program project are reported to be substantial. Results such as these are encouraging that the ICIDR program is fulfilling Program goals.

2. Tulane University, New Orleans, with Dr. Thomas Orihel as Principal Investigator, is collaborating with COLCIENCIAS (Colombian Fund for Research) in Cali, Colombia, to develop a research program which will address major public health problems of this neotropical region. The emphasis of the research effort is directed toward basic biological and epidemiological investigations of selected vector-borne parasitic diseases of man, in particular, filariasis and trypanosomiasis. Since this research was essentially an extension of the work previously funded under the ICMR program, the start-up period was relatively short, and their progress at the end of the 01 year is very good.

Filariasis surveys uncovered Dipelatonema perstans microfilaremias in 31% of the inhabitants in Guainia territory. The existence of perstans filariasis in the interior of Colombia holds considerable importance for the ICIDR program for the future since there will be an opportunity for investigations of various aspects of this filarial disease which are poorly understood.

To date, an enzyme-linked, immunosorbent (ELISA) has been developed to analyze levels of humoral immunity in serum of patients diagnosed parasitologically as having filariasis. Although the test appears promising for detecting filariasis in patients, further analyses and testing of additional antigen preparations must be done. In vitro lymphocyte blastogenesis has been tested in patients infected with W. bancrofti. Unfortunately, the peripheral blood lymphocytes did not respond uniformly. The reactivity depended on the stage of the disease. These results are consistent with the findings of other investigators. ICIDR investigators have succeeded in attracting grant support from the WHO and the Canadian International Development Research Center. Although the Center does not have a training component, it has served as a resource for graduate research studies. The research for two doctoral dissertations was carried out in Colombia and brought to successful conclusion during the past project year.

3. Harvard School of Public Health, Boston, with Dr. Thomas Weller as the Principal Investigator, is collaborating with the Universidade Federal da Bahia (UFBa) and the Fundacão Oswaldo Cruz (FOC) of the Federal Ministry of Health. With the arrival of three new full time scientists in Salvador last year, a multi-disciplinary team has been established representing the fields of epidemiology, clinical medicine, entomology and immunology. The administrative difficulties between Harvard and UFBa have been resolved, and the collaborative effort appears to be functioning very well.

The project laboratory continues to provide good serologic and parasitologic services. The ELISA test for antibodies to T. cruzi is being performed routinely. Dr. Todd plans to study lymphocyte transformation and suppression in chronic human Chagas' disease while supervising the base laboratory operation. Studies on the immunopathology of Chagas' disease in inbred mice is being carried out in Dr. Andrade's laboratory at FOC with the assistance of a technician supported by this project. The field area in Castro Alves, under Dr. Sherlock's supervision, remains the major site for field studies.

The new Avionics and American Optical electrocardiographic monitoring equipment is used on a daily basis in Dr. Guimares' laboratory in the Hospital Professor

Edgard Santos. Preliminary evaluation of the various methods of arrhythmia detection has shown the superiority of the 24-hour ambulatory monitor in detecting the maximal grade of ventricular ecotopic activity in Chagasic individuals.

4. Michigan State University, East Lansing with Co-Principal Investigators Drs. Jeffrey Williams and James Bennett, is associated with the Central Laboratories, Ministry of Health, Khartoum, Sudan, with the main field area located in Wau. This is the only group that had no on-going program with their foreign affiliate. Therefore, the Michigan investigators had to start from base zero in a very poor country where the black market flourishes and inflation runs rampant (ca 100%). However, the Government of Sudan welcomed the opportunity to collaborate with MSU and put-up some $40,000 to renovate laboratory and animal facilities in the Central Laboratories of the Ministry. The space is not yet available but will be in the near future. Because the central labs were not available, work was shifted to field operations. As a result, the group has been able to: complete one of the most successful clinical trials ever done in Sudan on a new antischistosomal agent, and initiate a second trial; establish a satellite malaria laboratory at the WHO Malaria Training Center in Sennar and set in place the in vitro cultivation of P. falciparum on site for characterization of the parasite and its sensitivity to chemotherapeutic agents and immune factors in serum; collect and collate unique clinical parasitological and epidemiological data on the nature of onchocerciasis in what appears to be one of the worst affected zones of river blindness in Africa; survey breeding sites of Simulium damnosum in water courses in the Wau study area, Bahr El Ghazal; and collect and preserve over 300 serum samples from patients with well-defined clinical entities of each of the major tropical diseases under study.

Collaborative arrangements have been made with a group funded by the Federal Republic of Germany, with the WHO-TDR investigators, and with US-AID representatives.

5. University of Illinois, with Drs. Kenrad Nelson and Victoria Schauf as Co-Principal Investigators, is collaborating with scientists at Chiang Mai University and McKean Rehabilitation Institute, Chiang Mai, Thailand. This collaborative research program consists of four interrelated projects: (1) a study of the epidemiology and sero-epidemiology of leprosy infection and disease, (2) an HLA genetic study of leprosy susceptibility, (3) a study of the immunoregulatory abnormalities and immunopathology of leprosy and its complications, and (4) a study of phagocytic cell functions and metabolism in leprosy.

This program project involves collaboration with investigators at the University of Illinois, University of Cincinnati, the University of New Mexico, Chiang Mai University, and the McKean Rehabilitation Institute. The collaborative effort appears to be functioning smoothly, and data are being collected and analyzed. Blood samples collected on newly-diagnosed cases for epidemiological studies are distributed to the various subcontractors for study. Dr. Bullock has found that there is a B lymphocyte hyperactivity in patients with lepromatous leprosy. Also, current data indicate that this may result from deficiency of T suppressor cell regulation over helper T cell activity and/or B cell function.

The grantee is making excellent progress studying a very difficult disease.

Part B (R-21) Exploratory/Developmental Grants

1. Columbia University, New York, with Dr. Philip D'Alesandro as the Principal Investigator, is collaborating with scientists at the Federal University of Rio de Janeiro, Brazil. An examination of cell surface carbohydrates of virulent and avirulent forms of Leishmania mexicana amazonensis was made using lectins of known specificity. Preliminary evidence with two of the purified lectins indicates that there is a difference in the surface antigens of the virulent and avirulent promastigotes. Binding studies with labelled lecitin are planned. Another project designed to study the relationship of parasite antigens to immunological dysfunctions associated with visceral leishmaniasis is in progress. To date, the investigators have been preparing reagents (antigens and antisera) and perfecting techniques to be used in the project.

Due to administrative difficulties, the grant start date was delayed, and the investigators are only now in the 02 year. Progress appears reasonable for this time period.

2. Ohio State University, Columbus, with Dr. John Briggs as Principal Investigator, is collaborating with scientists at the University of Nigeria, Nsukka. Dr. Briggs and his Nigerian collaborators are investigating biological control of vectors of human disease. The work is focused on isolating and screening spore-forming bacteria and fungi against mosquito larvae. To date, fungi isolates have been only weakly active against larval mosquitoes. Five of 33 spore-forming bacteria assayed caused mortality of both Aedes and Culex larvae. Only the isolate identified tentatively as Bacillus thuringiensis killed larvae at dilutions greater than 1-100. Work with this organism continues.

3. University of South Florida, Tampa, with Dr. Gary Rodrick as the Principal Investigator, is collaborating with scientists at Fundacão Oswaldo Cruz, Rio de Janeiro, Brazil. The investigator continues to study the spread of endemic schistosomiasis to the newly developed agricultural colonies in the interior of Northeast Brazil in the state of Piaiu. In addition, the disease has now been found in the states of Amazonas, Acre, Rondomia, and Matto Grasso do Norte. The study of possible snail vectors of schistosomiasis continues.

4. The University of Hawaii, with Dr. Wasim Siddiqui as Principal Investigator, is collaborating with his counterpart in Aligarh Muslim University in India. The investigators have succeeded in isolating and establishing a strain of P. falciparum from Aligarh in continuous culture. Also, calf serum and protease peptone have been substituted for human serum in the continuous culture medium. Dr. Siddiqui's project continues to attract and to stimulate Indian researchers in the field of malaria.

5. University of Washington, Seattle, with Dr. Thomas Buchanan as Principal Investigator, is collaborating with scientists at the National School of Biological Sciences, the National Polytechnic Institute, Mexico City. An arabinomannam antigen of mycobacteria was purified and used in an ELISA immunoassay. This assay was used to measure antibody in sera from leprosy patients and their contacts in Mexico. Some of the contacts of the leprosy patients had elevated antibody levels, indicating recent exposure to mycobacteria, possibly M. leprae. These patients will be evaluated further. The data will be used to develop criteria for predicting subclinical cases of leprosy.

The U.S.-Japan Cooperative Medical Science Program has been operative since 1965. The program provides the basis for collaborative research between scientists of the two countries on diseases of importance to Asia, although some are common to the United States and Japan as well as worldwide. For each disease or disease category a panel of scientific experts is appointed in each country to provide scientific advice and guidance. At present there are panels for the following: cholera, environmental mutagenesis and carcinogenesis, hepatitis (viral), leprosy, malnutrition, parasitic diseases (schistosomiasis and filariasis), tuberculosis and viral diseases (arboviruses, rabies, rotaviruses).

A very successful Immunology Symposium was held in Tokyo on October 14-15, 1980. The formation of an Immunology Panel was discussed at the Joint Committee meeting held the two days following the Symposium. The addition of immunology as a program area was approved in principle, but the name and guidelines were referred to the Subcommittee on Program Review and Planning for further deliberation. At the February, 1981 meeting in Honolulu, the Subcommittee recommended that the new area be known as the Immunology Board rather than a panel, since it was discipline rather than disease oriented, and was supported by the Ministry of Education rather than the Ministry of Health and Welfare. At the request of the Director of NIAID, the topic of how to curtail program spending, especially in the travel category, without sacrificing the mission of the Program was discussed. The Co-Chairmen of the Subcommittee agreed to seek advice from their respective Panel Chairmen and discuss this matter again at the next Joint Subcommittee and Committee meetings to be held in July, 1981 at the NIH. The discussions were held as recommended. It was agreed that changes would not be made unilaterally, since funding of the Program differs between the two countries. The Japanese depend upon the present configuration of this Program to obtain financial support. Mechanisms for implementing possible changes will be discussed at the next Subcommittee meeting, February 11-12, 1982.

The Joint Committee approved the formation of the Immunology Board along with its proposed membership and guidelines. The addition of immunology increases the number of program areas from eight to nine. The next meeting of the Joint Committee will be held in Tokyo on July 22 and 23, 1982. Immediately preceding this meeting, the Panels on Tuberculosis and Viral Diseases will hold symposia.

NIAID Collaboration in Africa currently consists of the ICIDR projects in Sudan and Nigeria noted above. During the year, the U.S.-Nigerian Joint Task Force on Health Cooperation agreed that cooperative activities will proceed in the area of biomedical research, with endemic communicable diseases of Nigeria being given highest priority. MIDP's role in this collaboration is evolving.

In December, 1980, Drs. Beck and Jordan visisted Brazzaville, Congo, and Nairobi, Kenya, while attending an International Conference on the Impact of Viral Diseases on Developing Countries. Their trip reports assess the prospects for collaborative research in these two countries.

BACTERIOLOGY AND VIROLOGY BRANCH

The Bacteriology and Virology Branch is responsible for the administration of a broad-ranging program of biomedical research; it supports program project grants, individual research grants, training grants, career development awards, individual postdoctoral fellowships, and contract programs of targeted research in bacteriology, virology, and mycology. Dr. Milton Puziss administers the Branch and the Bacteriology Program. Dr. William P. Allen administers the Virology Program, and also serves as Executive Secretary of the Panel on Viral Diseases of the U.S.-Japan Cooperative Medical Science Program. Dr. Darrel D. Gwinn administers the Mycology and Mycobacteriology Programs, and also serves as Executive Secretary of the Leprosy Panel and the Tuberculosis Panel, U.S.-Japan Cooperative Medical Science Program.

Approximate Level of Support

Bacteriology and Mycology

Activity	Number	Amount
Research Grants	228	$18,671,793
Research Program Projects	7	2,669,573
Research Contracts	8	709,531
Career Awards	9	337,215
Training Grants	16	1,049,110
Fellowships	6	107,480
Total	274	$23,544,702

Virology

Activity	Number	Amount
Research Grants	206	$19,848,492
Research Program Projects	2	705,509
Research Contracts	2	197,950
Career Awards	15	529,599
Training Grants	14	1,305,998
Fellowships	23	407,321
Total	262	$22,994,869

Branch

	Number	Amount
Total	536	$46,539,571

Program Summary

The program of this Branch focuses upon projects in both fundamental and applied biomedical research, with the ultimate and most significant objective being the translation of the knowledge gained into better and more practical methods for the diagnosis, prevention and therapy of bacterial, fungal and viral diseases. Sexually transmitted diseases and hospital associated (nosocomial) infections are programs of significant interest to the Congress and to the Institute. The biology and biochemistry of viral infections of humans and animals continue as subjects of considerable research interest. The focus of this new research is on the mechanisms of viral replication and the regulation of expression of gene products, the relation of viral structural components to function, and the immune responses to infection. Pathogenic mechanisms of acute viral diseases of man and mechanisms by which viruses can initiate and sustain persistent infections are areas of special concern and attention. Under the auspices of the U.S.-Japan Cooperative Medical Science Program certain disease areas have been singled out for special attention. These include research programs on tuberculosis and leprosy, as well as rabies, dengue and other arthropod transmitted diseases, infections that are still common in the world's developing countries.

Bacteriology

Sexually Transmitted Diseases (STD)

The sexually transmitted diseases constitute a major public health problem. No significant lessening of the overall problem is seen, although a slight leveling off of the gonorrhea rate has been noted. This disease continues as one of the major areas of concern in STD, although it may well be superseded, according to many experts, by an alarming increase in herpes simplex type 2 (HSV-2) infections. Nongonococcal urethritis (NGU) also shows a sustained high level of infection in the population. Firm data are lacking, however, in these as in all other STD problems. A significant increase in genital herpes is of concern to public health officials because of the potential for serious damage or death to infants born of mothers infected with herpes at the time of delivery. There is also increasing concern regarding other diseases, such as hepatitis, amebic dysentery and shigellosis, that are now being seen with increasing frequency, particularly among homosexual groups.

Research Highlights

AI 15113-03 A. H. Fieldsteel (SRI International): This investigator has been attempting the *in vitro* cultivation of *Treponema pallidum* - an elusive goal ever since the discovery of this causal agent of syphilis. A tissue culture system of cottontail rabbit epithelial cells (S_f1Ep) was selected because of consistently good treponemal attachment. A culture medium was developed containing fetal bovine serum and reducing agents. Growth of treponemes increased to about $3X10^6$ cells after six days, with motility averaging 76%. Peak increases appeared between seven and nine days; in one instance an eightfold increase, with motility of 89% was observed. These treponemes retained full virulence for rabbits, with lesions seen after inoculation of as few as 90 treponemes. Biochemical studies underway suggest that DNA increases equivalent to treponemal numbers are occurring in the S_f1Ep cells. If these studies can be independently verified by others, they may well open the way to development of a vaccine for syphilis. This is an exciting possibility for control of this disease of major significance in the underdeveloped world.

AI 13488-05 Z. A. McGee (Vanderbilt University): Previous studies described damage to human fallopian tube mucosa by low concentrations of purified gonococcal lipopolysaccharide (LPS). Recent data revealed that this damage could be blocked by polymixin B, a known inactivator of the lipid A portion of

LPS. Lysis of the gonococcal cell was found to be unnecessary for release of LPS; intact cells appeared to release small blebs from their surface that most likely contained endotoxin. The gonococcal LPS was species-specific for human fallopian tube mucosa only; this may be due to specific receptor sites on human but not animal oviduct mucosa. Chimpanzees were the only primates to develop an infection when gonococci were injected directly into their oviducts. The course of the infection in chimpanzee oviduct organ cultures was similar to that in human tubal organ cultures.

P01 AI 12192-06 K. K. Holmes (University of Washington): This complex program project (STD center) is one of three supported by this Institute. Recent data from one of the subprojects showed the successful adaptation of a coagglutinating (COA) technique for gonococcal typing. Three major groups, WI, WII and WIII based on gonococcal Protein I, were the predominant types. Good correlation was found between the COA and ELISA serotyping system. Data from this study showed that infection with one serotype conferred immunity to salpingitis re-infection with the same type. In another study specific DNA sequences of both the gonococcus and *Chlamydia trachomatis* were exploited as probes to identify organisms at low concentrations in clinical material. This method may have value for epidemiologic studies. Other clinical data indicated that risk of chlamydia infection in women was increased in younger age groups and in those who use oral contraceptives. *C. trachomatis* infection also was significantly associated with cervical cytological atypia. Data from a pilot study demonstrated the susceptibility of the pigtailed monkey to chlamydial salpingitis; this opens new avenues for detailed immunopathologic studies on chlamydial salpingitis and tubal disease. Studies on nonspecific vaginitis (NSV) revealed that continuous metronidazole therapy is the method of choice for treatment of this condition. IUDs were associated with increased risk of NSV as well as acute pelvic inflammatory disease and anaerobic infections. Other data showed that the presence of diamines (cadaverine and putrescine) in vaginal fluid was strongly correlated with NSV infections. This method can serve as a useful diagnostic tool to study NSV.

P01 AI 16959-01 J. C. Hume (Johns Hopkins University): One subproject in this program project deals with the biology of genital tract papilloma virus. Recent results indicate that the immunologic PAP technique was three times more likely to detect the virus in papillary lesions or flat cervical lesions than was thin-section electron microscopy. Among patients with childhood onset, 18% of specimens were antigen positive; no antigen, however, could be detected from patients with adult onset. A purified low MW protein has been obtained from viral DNA of flat condylomas and papillary lesions; it is believed to be DNA from the human papilloma virus-G (HPV-G). The ability to identify antigen in human tissues is an important advance in study of this STD problem.

N01 AI 92618 T. M. Buchanan (University of Washington): A vaccine for gonorrhea could serve as a major weapon in the ongoing effort to control this disease. Dr. Buchanan is continuing the development of candidate vaccine materials that hold great potential for eventual use in an acceptable vaccine. His recent research has led to identification of a fragment cleaved from the gonococcal pilus subunit that contains the binding site for attachment to human cells. This may become the prime candidate to induce protection against infection by gonococci. Other data indicated that one major serotype of protein I (the principal gonococcal outer membrane protein) accounts for over 90% of strains causing disseminated gonococcal infection. Newer methods have been developed for obtaining the protein I fraction in purer form and in higher concentration. This material is currently undergoing evaluation in volunteers in Phase I vaccine trials.

Hospital Associated Infections (HAI)

Hospital associated, or nosocomial, infections are a major public health problem in the U.S. Although the data are not firm, indications are that these infections result in significant mortality and prolonged hospitalization; excessive health care costs are the inevitable result. This problem will continue to increase as the immunocompromised segment of the population increases. The primary microorganisms involved are those of the gram negative groups; many of these are merely opportunistic invaders. There is a disturbing trend to antibiotic resistance among these organisms, thereby causing continued problems in patient therapy.

Research Highlights

AI 16108-02 E. J. Ziegler (University of California, San Diego): *Pseudomonas aeruginosa* infection causes a severe necrotizing vasculitis in burn patients; bacterial accumulation in blood vessel walls is seen without an inflammatory cell response. Vessel walls are destroyed, with infarction of surrounding tissue. This process may spread to skin, lungs or other distant sites as a consequence of bacteremia. In a rabbit model closely resembling human disease, it was shown that none of the extracellular products of *P. aeruginosa* was exclusively responsible for lethality; development of vasculitis is unrelated to elaboration of these well characterized proteins. A striking diminution of virulence was seen in pseudomonas mutants with loss of flagella, suggesting that motility may enhance invasiveness. Other data indicate that a continuing tissue blood supply is essential for vasculitis while identified properties of this bacterial species also may play a role in this disease.

AI 14032-04 R. L. Simmons (University of Minnesota): Intra-abdominal sepsis with or without abscess formation remains a difficult clinical problem. A fuller comprehension of this disease in a clinical setting is needed. The interaction of microbial pathogens in the peritoneal cavity with various cellular and molecular components of the host is of paramount importance. Data from this study demonstrated that introduction into the rat peritoneal cavity of 0.5% bovine fibrin clots containing high concentration of *E. coli* or *S. aureus* reduced the 24-hour mortality rate from 100% to 0% compared to controls infected with bacteria in saline solutions. It was further demonstrated that bacterial binding to the fibrin clot was inversely proportional to the initial contration of fibrinogen; *S. aureus* was bound to the clot to a greater extent than was *E. coli*. Human fibrinogen contains a specific binding site for staphylococci. These data lend further credence to the initial protective effect of fibrin in bacterial peritonitis. Other studies, in a rabbit model, revealed that lymphatic clearance of intact bacteria from the subcutaneous tissue is negligible during the initial and decisive period of soft tissue infections. Late clearance *in vivo* is consistent with the time needed for leukocyte infiltration to the infection site.

AI 14789-04 W. R. McCabe (Boston City Hospital): Gram negative bacteremia may be responsible for as many as 100,000 deaths annually in the U.S., according to

Dr. McCabe. He is currently attempting to develop a vaccine to overcome this problem; the vaccine is based on the Re mutant of Salmonella minnesota that will protect against heterologous gram negative infections. Recent data demonstrated that a modified killed cell vaccine given to 68 volunteers reduced the rate of adverse reactions markedly. Antibodies were induced that were highly protective for mice against lethal challenge with either heterologous bacilli or purified LPS. Results indicate that three daily injections in volunteer subjects induce a more rapid rise in protective antibodies - this is significant for potential clinical use. An ELISA test also was developed for measurement of IgM and IgG Re-specific antibody; levels of Re antibody of the IgM class were shown to correlate closely with mouse protective activity. Preliminary screening of some of the plasma units at the Massachusetts Department of Health Laboratories revealed that 5% of these units contained antibody levels to the Re mutant with titers greater than 1:80. These high titer plasma units afforded significant protection to mice challenged with different gram negative microorganisms.

AI 12936-05 J. W. Alexander (University of Cincinnati): Nosocomial infections continue as a major unsolved problem in surgical practice. The predominant immunologic defect predisposing to infection was shown to be defects in phagocytic function; malnutrition may also be a compounding factor. Data from animal studies revealed that administration of Corynebacterium parvum vaccine as an immunomodulator had a direct benefit in survival from burn injury. A synthetic immunomodulator (Pfizer, C.P. - 20,961) also showed good protection in mice challenged with a wide variety of bacteria. Preliminary study with small numbers of severely burned human patients further support this mode of therapy. This agent, however, appears to be unsuited for transplant patients because of shortened graft survival, as shown by studies of transplant survival in a dog model. Burn patients showed only mild, transient reactions to the administration of C. parvum; the results, however, were highly encouraging in preventing burn wound sepsis, but suggest the drug is of little benefit to a patient who is already septic.

AI 15826-02 R. D. Berg (Louisiana State University): Infection is recognized as a major cause of morbidity in patients with malignant disease. This project is on factors that promote or hinder bacterial translocation from the gastrointestinal (g.i.) tract and the role of the bowel flora in controlling translocation. In a mouse model it was demonstrated that there are mechanisms inhibiting translocation of certain indigenous bacteria (E. coli, lactobacilli) from the g.i. tract to the mesenteric lymph nodes, spleen and liver. Some antibiotics disrupt the ecological balance in the bowel, allowing certain bacteria to overpopulate the intestines and translocate to the mesenteric lymph nodes. T-cell dependent immunity also inhibited this translocation. Immunosuppressive agents, on the other hand, increased the incidence of bacterial translocation, thereby helping to establish infection.

Streptococcal Diseases and Sequelae

Streptococcal diseases and their sequelae continue as a troublesome health problem, particularly among the lower socioeconomic populations. The chief streptococcal disease problem in the U.S. is now group B streptococcal (GBS) infections; these are a significant cause of neonatal and infant mortality and morbidity. Group A streptococcal infections and their serious sequelae, rheumatic fever and acute glomerulor nephritis, although now somewhat less common in the U.S. and Europe, are nevertheless an important problem in many developing countries.

Research Highlights

AI 13249-06 C. J. Baker (Baylor College of Medicine): Group B streptococcal (GBS) disease among neonates and young infants constitute a significant problem in this age group. In a collaborative study, antibody assay by a number of different techniques (radio antigen binding, chick embryo assay, and indirect immunofluorescent) showed good correlation in measuring antibody to type III GBS antigen; both assays correlated with *in vivo* immunity. Countercurrent immunoelectrophoresis also appeared to be a useful diagnostic and prognostic tool for infants with GBS infection provided admission specimens from more than one source (urine, serum, CSF) were examined. Significant correlation was seen between low maternal antibody levels and neonatal early onset infection. In surviving infants convalescent sera indicated a poor antibody response to infection. These data extend earlier observations regarding a correlation between low levels of type-specific antibody in serum and risk of systemic infection with type III GBS.

N01 AI 72539 R. M. Swenson (Temple University): An ELISA assay for measuring antibody levels to type III GBS has been developed and has given excellent linear results that correlated with nanogram amounts of antibody bound by the quantitative precipitation technique. The assay system has now been automated for use with human acute and convalescent sera from patients with type III GBS disease. A similar ELISA assay using type II antigen has also been developed. Volunteers immunized with a type III GBS candidate vaccine responded with high antibody titers ranging up to 904 micrograms/ml. The type II GBS candidate vaccine also has been purified and tested for safety; no pyrogenicity or toxicity was observed in test animals. The antigenic effect is currently being determined in volunteers. A neonatal rat model of GBS disease is currently under development: preliminary data reveal that an LD_{50} for GBS strain M732 is less than 12 bacteria inoculated; anti-type III antisera protects against challenge with type III organisms.

AI 11822-08 V. A. Fischetti (Rockefeller University): Information regarding the structure and immunochemistry of type A streptococcal M protein is important for a fuller understanding of the pathogenicity of streptococcal infection. Results from this study have more firmly established the close relationship between streptococcal M protein and mammalian tropomyosin. This close relationship (especially in regard to the seven-residue periodicity found in both these molecules) strongly suggests that the M protein exists as a coiled coil structure on the streptococcal cell wall. This type of structure may help to determine the mechanisms by which the anti-phagocytic property of the molecule functions. The close structural relationship between M protein and tropomyosin may explain the etiology of cross-reactive antibody found in rheumatic patients. Immunologic studies also revealed that the Fc region of an opsonic antibody to M protein is required for phagocytosis of the group A streptococcus.

AI 06487-17 D. W. Watson (University of Minnesota): This investigator is continuing his studies on the pathobiology and immunology of the group A streptococcal pyrogenic exotoxin (SPE) and its role in the sequelae of group A streptococcal infections. Three toxin types, A, B, and C, have been isolated; a sensitive ELISA assay has been developed to determine antibody levels to these toxins. Attempts are underway to correlate the antibody levels as a measure of the immune response to HLA type - this is important in the kidney transplant program. SPE exotoxin was produced by group A strains isolated from patients in different countries; it appears to be a worldwide phenomenon. The toxin inhibits RNA synthesis and is a potent mitogen for T-cell lymphocytes. Other data show that bacteriophage plays a role in toxin elaboration; both types A and C SPE were readily transferred from toxigenic to nontoxigenic strains by lysogeny. It is possible that SPE B-toxin is expressed by chromosomal genes. Preliminary studies with experimental lots of human immune globulin have shown the presence of high titers of anti-SPE antibody. If these preparations can protect passively against toxic properites of SPE they might be used therapeutically in complicated group A streptococcal infections.

AI 13150-05 H. R. Hill (University of Utah): In a neonatal rat model of GBS infection, strains of types Ia, II, and III were evaluated for virulence and for correlation with their capsular polysaccharide content. No correlation was detected between capsular content and resistance to opsonization or virulence. Other studies with this model revealed that neutropenia and neutrophil storage pool depletion are prominent features of experimental type III GBS infection. The data suggest that an impaired membrane function may contribute to neonatal PMN abnormalities. An ELISA test also was developed for GBS types II and III antibodies; antibody in human sera against intact bacteria correlated with opsonic antibody activity (measured by chemiluminescence assay). A population of Mexican-American women is under investigation to determine if prevalence of a different antibody could account for the low rate of GBS disease in their offspring. Preliminary results suggest that these women have a much higher prevalence of types II and III antibody as measured by ELISA.

Other Disease Problems

There are many other problems of bacterial diseases that merit attention. Many of these bacterial infections, once considered of little significance because of the widespread availability of antibiotics, remain as important diseases. Certain infections are now known to be more complex in their disease manifestations, while others have only recently emerged as significant problems.

Research Highlights

AI 16121-02 R. D. Miller (University of Louisville): Isolated cases and outbreaks of Legionnaires' disease still occur periodically, with significant morbidity and mortality. Transmission of the etiologic agent in many cases is obscure. Clearer understanding of the biology of this microorganism could be of value in delineating the natural habitat and the conditions of transmission to humans of this pathogen. Dr. Miller reports the development of a synthetic medium for growth of *Legionella pneumophila* containing eight required amino acids and inorganic salts. Study of extracellular enzymes produced by this organism showed the presence of proteases, lipase, acid phosphatase, DNA and RNAases, and beta-lactamase. This may yield clues as to the virulence factors and pathogenic mechanisms of the microorganism. The *Legionella* bacterium showed good survival at 4°C or 25°C. Cells at 37°C showed markedly diminished survival; this was a function of pH, with better survival at pH 6.0 than at higher pH levels.

AI 15903-02 H. N. Beaty (University of Vermont): Antibody response to intratracheal instillation of *L. pneumophila* was measured in a series of animal models by IF and agglutinin tests; excellent correlation between these methods was obtained. In guinea pigs a fourfold rise in antibody could be detected three days post-infection; no positive serologic response, however, was seen using heat-killed *L. pneumophila* as controls. Pneumonia was induced in the guinea pigs by the instillation method; with an inoculum of 10^5 CFU, 50% of infected animals died of fulminant pneumonia, with a peak mortality seen at four days. Animals surviving seven or more days had decreased bacterial counts in their lungs. Hamsters showed an uncharacteristic hemorrhagic component to their infection, while rats developed evidence of pneumonia but were much more resistant than guinea pigs to the lethal effects of the infection. These animals may therefore be much closer to the human situation and could serve as an effective animal model of infection.

AI 16557-01 M. J. Thomassen (Case Western Reserve University): Chronic pulmonary infection and associated airway obstruction are major factors in the pathophysiology of cystic fibrosis (CF). Data from this study demonstrated the presence of an activity of CF serum that specifically inhibits phagocytosis of *Pseudomonas aeruginosa* by both normal and CF alveolar macrophages (AM). This investigator established that, despite a chronic lung infection process, AM cells from CF patients morphologically and functionally resemble cells from normal individuals. Activated cells were found to be unaffected by the phagocytic inhibitory activity present in CF serum. This failure of the AM to become "activated" may contribute to the persistence of the pseudomonas, resulting in further progression of CF lung disease. Study of the ability of PMNs and monocytes from normals and from CF patients to phagocytize pseudomonas organisms showed that cells from either of these population groups have comparable phagocytic ability in their serum.

AI 15336-03 H. Sugiyama (University of Wisconsin): Infant botulism differs from classic food poisoning botulism in affecting only the very young. Additionally, the causative toxin is not formed in food but in the gut during multiplication of the *Clostridium botulinum* spores. Dr. Sugiyama is developing a mouse model for studying botulism of human infants in attempts to understand why this intoxication is restricted to infants. *C. botulinum* spores were administered intragastrically to 7-13 day old mice and infant rats. Botulinum toxin was produced in the lower gut, but not when the spores were given to older animals. The different gut flora of infant and adult animals is the most likely reason for the contrasting susceptibilities of the different age groups. Germfree adult rats and mice are highly susceptible to this intoxication. When germfree adult mice are allowed to acquire the intestinal flora of the normal adult animal, they become as resistant to the infection as conventionally reared animals. The normal intestinal flora probably competes with and prevents multiplication of the pathogenic clostridia in the gut. It was also revealed that resistance to botulism by bacterial competition does not require many different species. These studies generated additional evidence that the restricted age incidence of infant botulism has a microbial ecological basis, whereby multiplication of the pathogen is controlled by the normal intestinal flora.

Meetings

1. The Bacteriology and Virology Branch, in conjunction with the Development and Applications Branch, MIDP, co-sponsored a concept review workshop in January 1981 on the "Potential of Human Immune Globulin for the Prevention and Treatment of Bacterial Diseases." The conferees agreed that a program for the development of human immune globulin is needed and that the NIH should encourage this development.

2. The Bacteriology and Virology Branch, MIDP played a leading role in developing a Conference on Bacterial Virulence and Pathogenesis, held in July 1981 at the Rocky Mountain Laboratory, Hamilton, Montana.

Leprosy

Current research in leprosy is focused on bacteriology, chemotherapy, and immunology. Bacteriological research revolves around the fact that Mycobacterium leprae has never been cultivated in vitro. Chemotherapeutic study has involved screening of drugs against M. leprae in a mouse footpad model, studies of the action of drugs in mice and in man, and investigation of drug pharmacokinetics. Immunological investigations focus on new diagnostic skin tests; blood tests are being developed that may be useful in detecting early infection. All these research efforts are directed at obtaining a greater understanding of this microorganism and the development of new methods of treatment and control of the disease.

Research Highlights

N01 AI 92625 P. J. Brennan (National Jewish Hospital, Denver): The major thrust of Dr. Brennan's work is to isolate, purify, and characterize species-specific surface antigens from the purified leprosy bacillus obtained from infected armadillo tissue. Preliminary experiments cited previously indicated that M. leprae contains species-specific lipid antigens. In more recent experiments, chemical analysis of the glycolipid showed the existence of a phenolic nucleus. Further investigation showed the presence of a long chain β-glycol substituent to which was attached two fatty acyl functions. An examination of the glycolipid sugar composition revealed that it contained three sugars, some of which had not previously been encountered in nature. Thus, M. leprae contains a phenolic glycolipid, the sugar composition of which is unique and is probably exclusive to M. leprae.

AI 08417-11 A. H. Fieldsteel (Stanford Research Institute): One aspect of Dr. Fieldsteel's research involves utilizing the neonatally thymectomized Lewis Rat (NTLR) as a model of lepromatous leprosy for the development of effective chemotherapeutic regimens. The primary goal is to investigate these regimens for their ability to eradicate M. leprae infection. He has developed several regimens involving combinations of rifampin and dapsone as well as regimens involving continuous treatment with rifampin or clofazimine. The NTLR responds to these drug regimens in a manner very similar to that of human lepromatous leprosy patients. The NTLR model is approximately 100 times more sensitive in the detection of bacterial persistence in long term therapy than the mouse footpad model.

Tuberculosis

Tuberculosis remains one of the most important infectious diseases of mankind; it is especially prevalent in the underdeveloped countries. Transmission of tuberculosis depends on the degree to which a diseased person generates infectious aerosols, the intensity and duration of exposure to the ill person, and the susceptibility of the host. Most patients with tuberculosis are not highly contagious, although an occasional patient may be. The period of maximum infectivity is likely to be just before diagnosis, when the patient's cough and other symptoms are usually most severe. Since the introduction of mutiple-drug chemotherapy about 30 years ago, the recommended duration of daily chemotherapy for tuberculosis has been 18 to 24 months. Following the introduction of rifampin a few years ago, important clinical observations were made on the use of rifampin combined with isoniazid in a short course of therapy. The present regimen of choice for newly discovered cases of tuberculosis is nine months of treatment with isoniazid and rifampin. During the past 25 years the number of reported cases of tuberculosis in the United States has decreased approximately 4% per year; however, beginning in 1979 the number of reported cases leveled off at approximately 28,000 per year.

Research Highlights

AI 11807-04 B. M. Sultzer (Downstate Medical Center, State University of New York): It has been shown that tuberculin can activate B-lymphocytes in a nonspecific fashion as well as stimulate both B and T-cells as a specific antigen. This is of particular significance relative to understanding the complex cellular reactions of the host to the tubercule bacillus. Dr. Sultzer has shown that suppressor T-cells are produced in the spleens of BCG infected mice. The suppressor cell activation by BCG infection is an example of regulation by one cell type over another during the infective process.

AI 11297-08 K. Takayama (Veterans' Hospital, Madison, Wisconsin): This group is studying the mechanism of action of ethambutol (EMB) on Mycobacterium smegmatis. They have found that EMB stimulates the synthesis of trehalose monomycolate (TM) after about one minute of exposure; it also causes trehalose dimycolate (TD) and "free" mycolic acid to accumulate and inhibits the transfer of mycolic acid to cell wall, all after 8-12 minutes. EMB also causes the cellular leakage of TM, TD, mycolic acid, and phospholipids after 30 minutes of exposure. Cell death occurs after about 4 hours of exposure. It is clear that EMB affects some aspects of utilization of mycolic acid at a very early exposure time.

AI 13813-03 H. Gruft (New York State Department of Health): In previous studies, Dr. Gruft was able to show that the distribution of Mycobacterium avium-intracellulare-scrofulaceum complex (MAIS), along the east coast of the U.S. was related to the salt concentration in the water as well as the section of the country studied. He reasoned that individuals became infected by inhaling mycobacteria contaminated water droplets escaping from these estuarine waters. The question that remained was, what was the source of these mycobacterial contaminants? Recently he investigated a few regions in greater detail and isolated MAIS strains from soil near the coastal water sites as well as from pine mulch used for chicken litter at concentrations greater than he had detected in the estuary waters. These results suggest a movement, especially for M. intracellulare, from the soil and/or pine mulch, to the water, to the air, to a susceptible host.

AI 15102-02 T. M. Daniel (Case Western Reserve University): Recent reports have suggested that patients with recently diagnosed tuberculosis present an immune spectrum similar to that seen in leprosy. Dr. Daniel studied the immune responsiveness to mycobacterial antigens of 65 patients with pulmonary tuberculosis using delayed skin test reactions and enzyme-linked immunosorbent antibody assays. Evidence for a spectrum of immune responsiveness was found. Six of 35 patients studied during the first month of therapy had tuberculin skin test anergy. Anergy was not related to state of nutrition or extent of disease, but it may have been associated with radiographically acute disease. The anergic patients had somewhat higher antibody titers to mycobacterial protein but not to polysaccaride antigens, than did the nonanergic patients.

Mycology

Techniques for the isolation and identification of fungi from clinical specimens have been well delineated and are available in most hospital laboratories. Inherent problems which make diagnosis of mycotic infections difficult are the time required for the growth (can be weeks) of a potential pathogen and the time required to identify positively the organism once colonies appear. Often a positive diagnosis of mycotic infection is made postmortem. There is a need for more rapid and specific diagnostic adjuncts in fungal infections. The absence of recognised reference materials and procedures makes comparison of the various independent studies difficult. The general belief among investigators in mycology is that many more people are suffering from systemic mycotic infections than are being diagnosed.

Research Highlights

AI 14061-02 D. Pappagianis (University of California School of Medicine, Davis): Dr. Pappagianis is studying the fate of Coccidioides immitis following phagocytosis by murine peritoneal macrophages. He found that the maximal adverse effect against C. immitis through phagocytosis and killing occurs only when macrophages are influenced by immune splenic lymphocytes. Neither cell type alone is capable of killing the fungus. Thus, splenic lymphocytes from immune mice stimulate macrophage mediated killing of C. immitis in vitro.

AI 16434-02 D. J. Drutz (University of Texas Health Science Center): The objectives of this research are to determine the site and mechanism of Candida albicans localization in the kidney and to define the mechanism whereby the fungus moves from the peritubular vasculature to the renal tubular lumen. The studies indicate that the localization of C. albicans in the renal vasculature is based on a physical affinity between endothelial cells of peritubular capillaries in the cortical area and of C. albicans. These studies prove that localization is not based on size, and is not a universal attribute of Candida species. Tubular penetration does not appear to relate to endothelial or tubular destruction in the early phases of infection, nor is endothelial or tubular cell endocytosis of fungi seen. Instead, fungi seem to pass through or between these cell types by a mechanism that involves germ tube formation.

AI 15682-03 J. M. Jones (University of Wisconsin Medical School): Dr. Jones earlier reported on the development of a radioimmunoassay (RIA) to measure levels of serum antibody (Ab) against Candida cell wall mannan (CWM). He has now developed an RIA to detect Ab against an ammonium sulfate-soluble fraction of a cytoplasmic extract (CYE) of Candida. He found that the sulfate-soluble fraction of CYE contained a major cytoplasmic antigen, the most prominent component of CYE. Recently he developed an enzyme-linked immunosorbent assay

(ELISA) to measure anti-CWM Ab and to detect CWM in serum. The ELISA has the same sensitivity as the RIA, but can be performed more rapidly using relatively simple reagents and equipment.

AI 16567-02 P. T. Magee (Michigan State University): Dr. Magee's overall objective is to establish a genetic system to use in exploring the genetic basis of pathogenicity. His early observations, that auxotrophs tend to occur as sectored colonies, led him to hypothesize that the organism is functionally diploid. He was able to use this observation to demonstrate heterozygosity, since if new phenotypes occur because of mitotic crossing-over, the sector bearing the wild-type phenotype should no longer give rise to the mutant phenotype. In seven isolates, he confirmed the heterozygous state of *C. albicans*. This method will allow the construction of mutants altered in various parameters thought to be important in pathogenicity; such mutants can be tested directly in animal models.

Meetings

A workshop on the "Serodiagnosis of Systemic Mycoses" was held June 18-19, 1981. Fifteen papers were presented on this subject with active discussion following each presentation. The participants also discussed gap areas and research needs in the serodiagnosis of systemic mycosis and developed recommendations for future research.

Virology

Biology of Viruses and Viral Diseases

Because of public concern expressed over diseases caused by herpesviruses considerable attention is being focused on herpesvirus research. Genital herpes research has been singled out as part of the Sexually Transmitted Diseases Program deserving special attention. Research projects associated with viral diseases of interest to the U.S.-Japan Cooperative Medical Science Program are also identified for review.

Research Highlights:

AI 15488-03 N. Frenkel (University of Chicago): The genetic material of herpesviruses is packaged into a relatively large double strand of DNA. Understanding the structure and function of the DNA during replication is difficult to approach because of its complexity. Dr. Frenkel has successfully devised an approach to study the DNA of herpes simplex virus (HSV), types 1 and 2, the agents of oral and genital herpes, by using defective viral particles. The DNA of the defective particles are useful as tools in dissecting and mapping the DNA of complete virus. Thus far, she has grossly mapped the location of one HSV replication origin within one component (S) of the genome and provided evidence for finding the origin of replication of the other component (L). She has also identified new intermediates of DNA replication that are important for the assembly and packaging of new virus particles and produced new evidence for the rolling circle mechanism of DNA replication. This information is important and critical to: (i) the eventual design of new attenuated HSV viruses that can be considered safe for live vaccines and (ii) the targeting of antiviral compounds for treating herpes infections.

AI 02032-24 D. J. O'Callaghan (University of Mississippi Medical Center): Dr. O'Callaghan has been using herpesviruses of horses as models for understanding the functions of specific genes. The equine herpesviruses are ideal models for studying persistent viral infections, their oncogenic potential, and other cell-virus interactions that are not easily studied with the human herpesviruses. Although herpesviruses are known to be associated with neoplastic disease in several animals and possibly in man, this propensity of the virus has been difficult to study in cell culture systems because most herpesviruses destroy the cells they infect when cultured. Dr. O'Callaghan has been able to overcome this obstacle by studying equine abortion virus (EHV-1), equine cytomegalovirus (EHV-2), and the equine coital exanthema virus (EHV-3). All three are transmitted venereally and cause venereal disease in horses, and at least one (EHV-2) causes tumors in hamsters as well as transformation in cell cultures. Dr. O'Callaghan has mapped the area of the genome of EHV-1 that is responsible for oncogenic transformation. He has found that this virus also produces a defective particle which contributes to its ability to establish persistent infections. By comparing the biochemistry of EHV-1 with that of other herpesviruses he hopes to identify gene products responsible for tumorgenicity and persistence.

AI 15644-03 - B. R. Francke (Yale University): Studies on virus-cell interactions have demonstrated that some mammalian cells allow herpes simplex virus (HSV) to penetrate, replicate and produce new virus particles. This is called a permissive infection. In non-permissive cells the virus fails to yield new progeny. In a series of experiments to identify the site of the block in non-permissive infections, Dr. Francke has demonstrated that in non-permissive Chinese hamster cells the HSV adsorbs to the cell surface, penetrates the plasma membrane, moves to and penetrates the nuclear membrane and initiates replication of parental DNA just as the virus does in permissive cells. The non-permissive infection, however, fails to turn off macromolecular synthesis by the host and only low levels of virus specific synthesis and no viral progeny are detectable. The block most likely prevents the efficient expression of viral genes at the transcriptional and/or translational level that is necessary for the final assembly of the virus particle. Dr. Franke's research is focused toward the identification of these gene functions.

AI 14981-04 B.T. Rouse (University of Tennessee): The immune mechanisms that influence herpesvirus infections are little understood. Of the several important herpesvirus diseases that afflict mankind, persistence of virus is a major factor; such viruses can be reactivated to give rise to recrudescent disease. Dr. Rouse has been investigating cellular immune responses of experimental animals to herpesvirus infections to determine which are involved in maintaining latency without recrudescence. It appears that components of cell-mediated immunity are most crucial in the immune defenses, especially the cytotoxic T lymphocyte (CTL). In detailed studies Dr. Rouse has found that CTL can be detected as early as 3-4 days after infection, that the response peaks at 6-7 days and then declines to undetectable levels by day 14. He has shown that this primary response is associated with genetically controlled histocompatibility antigens of the mouse host. The CTL response cannot be induced with purified viral glycoproteins nor with viruses inactivated by heat or detergents. This is consistent with the observation that killed virus appears to be poorly immunogenic. It was determined that failure of inactivated virus to induce a CTL response was not due to a loss in antigenicity but was due to the loss of a necessary stimulant for processing these immune cells. Elucidation of this immune mechanism is important to the design and evaluation of herpesvirus vaccines.

AI 15451-03 C.S.H. Young (Columbia University): Genetic recombination is a fundamental property of the genomes of most life forms. Its biological significance lies in the production of new combinations of genes during reproduction and the consequent expression of new types of life. The mechanisms by which recombination occurs are understood best in the case of DNA synthesis and mRNA translation. Dr. Young has been pursuing the process of genetic recombination through the use of double stranded DNA of adenoviruses. He has found that recombination of the viral DNA is a continous process occurring throughout much of the infectious cycle from some time after uncoating of the virus particle until encapsidation of new progeny virus. The process is not regulated by some viral gene products during early phases of infection but is complicated and obscured by the numerous events occurring during the expansion of the DNA pool. It was also determined that several sequential recombination events occur in the lineage of a DNA molecule and that recombination can occur in the absence of DNA synthesis and late viral functions. These studies are continuing to generate information about the enzymes and the structural proteins necessary for initiating recombination.

Workshop on the Concept of Genital Herpes Vaccines

Current information bearing on the concept of using vaccines to prevent or treat genital herpes was reviewed by staff and a small group of consultants in February, 1981. Recommendations and conclusions emanating from this workshop focused on the need for more reliable information on: the magnitude of the public health problem of herpes genitalis in the U.S. population; the natural history of the disease and its transmission; the indicators of immunity against infection, and disease; and the factors influencing reactivation of virus and recurrent disease. The partipants agreed that vaccines may help to prevent or lessen disease even if they fail to prevent infection. They recommended that development of vaccines should proceed, but not to the detriment of other approaches to control and treatment, such as antiviral drugs.

Virology Research under the Auspices of the U.S.-Japan Cooperative Medical Science Program: The goals for this program include the encouragement and support of research toward the eventual control of rabies, viral gastroenteritis and arthropod-borne diseases.

Research Highlights

AI 09706-10 H. Koprowski (The Wistar Institute): In June, 1980, the human diploid rabies vaccine, originally developed by the Wistar Institute, was approved for general use in the United States. Vaccine is available now only through state and federal public health agencies.

The study of rabies viruses and rabies virus antigens has been aided by significant technological advances: (1) Monoclonal antibodies of hybridoma origin have been used to investigate world-wide distribution of rabies virus strains. (2) In collaboration with several laboratories in Europe and the Americas the analysis of street rabies (wild type) virus isolates were initiated. Special attention was given to strains of virus obtained from individuals who died from rabies despite post-exposure vaccine treatment, or from animals who died in spite of prophylactic vaccination. Preliminary results have indicated that natural antigenic variants of rabies virus do exist outside the laboratory and that locally produced rabies vaccines conferred no, or only partial, protection. There is a strong indication that those strains were of bat origin. More information is needed before a decision can be made to select a new strain for vaccine production either alone or in combination with other strains in order to assure protection against field viruses present in a given geographic area.

AI 12541-06 - G.H. Tignor (Yale University): Infections of nerve axons have been observed during the course of rabies virus infection. Transport of the virus from distal sites of inoculation to the central nervous system via nerves has been postulated and tested. However, little is known about how the virus enters the nerves near the site of inoculation. Dr. Tignor's research has shed some new light on the entry of rabies virus at nerve terminals. He has confirmed that the virus does enter at motor end-plates faster than at sensory nerve endings. His data show that there is a specific attachment site for rabies virus and possibly other neurotropic viruses in the region of the nerve terminal. The data also suggest that the site may be related to the aceytylcholine receptors which are essential in the transmission of nerve impulses.

AI 15400-03 D.H.L. Bishop (University of Alabama in Birmingham): With RNA viruses that have segmented genomes it has been possible to produce reassortants of RNA segments in such a way as to determine which segments code for proteins essential for viral virulence. Dr. Bishop has investigated reassortants among 20 bunyaviruses representing 9 of the 20 known serogroups for this genus. It has been found that not all reassortants are formed efficiently during mixed infections. The reasons for these genetic limitations and the degree of compatibilities with other viruses are still being investigated. In collaborative studies with Drs. Beaty and Shope at Yale, the factor responsible for neurovirulence in mice and for vector competence in mosquitoes has been assigned to the M segment of the RNA genome. Dr. Bishop is pursuing the cloning of this segment for evential base sequencing and identification of the polypeptide products responsible for the neurovirulence characteristics.

AI 15426-03 B.J. Beaty (Yale University): The correlation of gene structure with biological function has been extended to the vector-virus relationships of the arthropod-borne viruses by Dr. Beaty. The aim of these studies is to elucidate the contribution of particular bunyavirus genes to the biology and transmission potential of the viruses. Using the LaCrosse virus (LAC), the agent of California encephalitis, and a related nonpathogenic virus, snowshoe hare virus (SSH) which reassorts with LAC, Dr. Beaty has shown that the M segment of LAC carries the determinant for vector transmission in *Aedes triseriatus* mosquitoes. There appears to be a partial block in the mosquito midgut to viral reassortants carrying the avirulence marker, but when the midgut is by-passed by intrathoracic inoculation the avirulent viruses do not infect the salivary glands as efficiently as the fully virulent LAC virus. Dr. Beaty has also demonstrated that reassorting of these viruses can occur *in vivo* in mosquitoes when they are dually infected with two related viruses. These results lend further credence to the potential of these viruses to reassort naturally and produce virus progeny with new vector preferences and pathogenic potentials. Such natural recombinations among related viruses could help to explain the already diverse spectrum of arthropod-borne viruses isolated from natural sources.

CLINICAL AND EPIDEMIOLOGICAL STUDIES BRANCH

The Clinical Studies Branch was reorganized into the Clinical and Epidemiological Studies Branch (CESB) in April, 1981. The Epidemiology and Biometry Branch was disestablished and incorporated into the CESB as the Epidemiology and Biometry Section. The CESB serves as the Institute's focus for the development and processing of Investigational New Drug Applications, and maintains liaison with the NIH Human Research Review Panel and the NIAID-Clinical Research Subpanel. It provides and monitors a closed clinical facility for Institute volunteer studies, and conducts, promotes and supports the development and evaluation of procedures for the diagnosis, prevention and treatment of infectious disease, particularly those procedures leading to improved patient care. The Epidemiology and Biometry (EB) Section conducts epidemiological research in infectious, allergic and immunological diseases, and analyzes data from various sources for trends in U.S. Vital Statistics in areas of interest to the Institute. The EB Section manages the Institute's program for mathematical and statistical studies, and provides biometrical consultation to other branches and laboratories. The CESB enlarged its extramural activities with the acquisition of a major portion of the enteric diseases program, which includes the coordination of the activities of the Cholera Panel of the U.S.-Japan Cooperative Medical Science Program.

Approximate Level of Support

Activity	Number	Amount
Epidemiology, Antibiotic Trials, Rapid Viral Diagnosis, Nutrition, Volunteer Facility		
Research Contracts	7	$1,552,244
Research Grants	6	$494,437
Training Grants	2	$25,618
Total	15	$2,072,299
Diarrheal Diseases		
Research Contracts	7	$223,971
Research Grants	27	$2,379,142
Program Project Grants	1	$308,479
Training Grants	1	$39,701
Fellowships	2	$46,116
Total	38	$2,997,409
Branch		
Research Contracts	14	$1,776,215
Research Grants	33	$2,873,579
Program Project Grants	1	$308,479
Training Grants	3	$65,319

Fellowships	2	$46,116
Total	53	$5,069,708

Program Areas

Enteric Diseases Program

With the departure of Dr. George Curlin, Dr. R. Edelman and Mrs. J. Smith were appointed Enteric Diseases Program Project Officer and Assistant Project Officer, respectively. One new Enteric Disease Study Center was established in FY '81 by Johns Hopkins University at the Whiteriver Apache Indian Reservation in Arizona. This center, approved for funding until FY '86, will study the epidemiology of diarrhea on the Reservation and the effect of potable water supply on enteric illness. The Enteric Disease Study Center at the University of Texas in Houston continued its long term serveillance of Day-Care Centers. There were outbreaks of diarrhea in four of the six day care centers under study. *Shigella* species were identified in five outbreaks, rotavirus was responsible for two outbreaks, and *Giardia* was identified in one. Mechanisms of transmission among children, their families, and day care center staff are under investigation. NIAID support for the Enteric Disease Centers at the Children's Hospital National Medical Center, Washington, D.C. and at the University of Michigan was terminated in FY '81. Analysis of epidemiological data on rotavirus disease in outpatients and families studied at these centers is underway.

Cholera

Factors which determine the distribution of *V. cholerae* and other vibrios in environmental water are now thought to be high salinity, nutrient concentration, association with copepods, and adsorption to chitin. Studies on the pathogenesis of cholera have pointed to the importance of the following factors: Cell-associated colonization factors and mucosal adherence appear to be essential virulence mechanisms for *V. cholerae*. Proteolytic enzymes, produced by both clinical and environmental isolates of *V. cholerae*, seem not to be critical virulence factors. Outer membrane proteins may be related to virulence, because these are frequently altered in virulence-deficient mutants. Some plasmids found in clinical isolates alter virulence in animal models; depending upon the specific plasmid, virulence may be either increased, unaffected, or decreased.

An improved adult rabbit model for study of immunity to *V. cholerae* and toxigenic *E. coli* infections has been established. In this model, prior colonization has been shown to protect against disease due to subsequent challenge with live *V. cholerae*; it should prove valuable for study of immunity to live virulent strains of *V. cholerae* or mutants (vaccine candidates) derived from them. Studies of dogs immunized orally with a crude toxin-containing culture filtrate of *V. cholerae* show this to be the best non-living oral immunogen yet studied in this model. Thus, holotoxin, in small amounts, may be safe and very effective as a component of an oral, non-living cholera vaccine. In addition the protective value of procholeragenoid, a heat-induced macromolecular aggregate of holotoxin, is being tested as a vaccine because it retains full immunogenicity after heating despite loss of 90-99 percent of its enterotoxicity. Studies of Texas Star, V. cholerae vaccine in volunteers is described under the section heading, "Closed Volunteer Facility." This strain has been shown not to revert to toxigenicity in mice, and to be a poor donor and recipient of genetic material. Studies of the immune response to naturally occurring cholera and to oral or parenteral immunization are being conducted among adults in Bangladesh. Parenteral immunization can boost a previously primed local enteric response, but better responses follow oral immunization.

Toxigenic E. coli

Intestinal colonization by toxigenic E. coli is mediated by pilus-like colonization factors (CFA's) on the bacterial surface. One laboratory believes three CFA's have been found on E. coli pathogenic for humans. Progress has been made in attempts to clone plasmid DNA that controls pilus production. The purpose is to produce avirulent (non-toxigenic) strains that produce immunogenic CFA antigen and thus might prove useful as live vaccine strains. Milk samples from Bangladeshi women showed anti-CFA I or II, or both, in 74 percent of samples. In contrast, mothers in Houston, an area of low incidence for E. coli diarrhea, had anti-CFA I or II in only 25 percent of milk samples, thus attesting to the immunogenicity of CFA in vivo. A typing system for putative CFA's on E. coli has been developed, using mannose-resistant hemagglutination of RBC's from various species. This provides a useful epidemiologic tool for rapid identification of potentially toxigenic strains. Characteristic hemmagglutination patterns have also been identified for enteropathogenic E. coli strains and for E. coli that cause urinary infections and bacteremia. Studies of a purified type I pilus vaccine in volunteers are reviewed under the section, "Closed Volunteer Facility."

Studies on E. coli ST toxin have shown it to be a structurally heterogeneous collection of toxins with molecular weights ranging from 1900-3600. At least three different ST's have identical biological effects. The nucleotide sequence controlling E. coli LT synthesis has been fully determined and shown to have close homology to cholera toxin. Additionally, the gene encoding the B subunit of LT has been isolated. Bacteria producing the B subunit may prove useful as live vaccine strains.

DNA fragments totally specific for ST and LT toxins have been isolated and used as probes by DNA hybridization to study the epidemiology of E. coli diarrheal disease. This test is simple, sensitive, and performed directly on stool samples under field conditions. The test detects ST or LT in coprobacteria grown on filter paper discs which are treated with the radio-labelled DNA probe. Virtually all ST and LT strains of E. coli are identified by the probe. This method has also shown that certain environmental strains of V. cholerae 01 have no trace of the toxin gene and hence cannot be diarrheagenic.

Closed Volunteer Facility

The CESB currently supports one research contract with the University of Maryland. This contract, recompeted this year and awarded to the incumbent for five additional years, maintains and supports the Center for Vaccine Development

(CVD) where volunteers are studied before and after they are challenged with potentially transmissible infectious agents and candidate vaccines. During the past year, the contractor performed 21 in-patient studies (247 volunteers) and 7 out-patient studies (195 volunteers). These studies were carried out under 10 active protocols approved by the Institutional Review Boards of the University of Maryland and NIAID. Included were *Escherichia coli* diarrhea (10 studies, 3 protocols); cholera (3 studies, 1 protocol); viral diarrhea (3 studies, 2 protocols); influenza (7 studies, 1 protocol); Rocky Mountain Spotted Fever (2 studies, 1 protocol); risk assessment of plasmid transfer in non-pathogenic *E. coli* (2 studies, 1 protocol); oral typhoid vaccine (1 study, 1 protocol). Highlights of these studies in volunteers are summarized below.

Fifty-six volunteers were immunized with two subcutaneous doses of a chick cell culture, inactivated, Rocky Mountain Spotted Fever vaccine developed by the U.S. Army. The vaccine caused only benign local and systemic reactions. Sixteen of these vaccinated individuals were challenged with virulent *R. rickettsii* one month after immunization, and 12 developed Rocky Mountain Spotted Fever. Six of six unvaccinated controls developed RMSF after identical challenge. Thus the vaccine was only 25% effective in preventing RMSF, though it did prolong the incubation period and attenuate the severity of the induced infection. Serological and rickettsemia data are pending.

An enteropathogenic (EPEC) strain of *E. coli* isolated from a sporadic case of diarrhea induced diarrhea in six of 11 volunteers. This study confirms the ability of EPEC to cause sporatic diarrhea in addition to nursery outbreaks, despite the fact that these strains are non-invasive and do not produce a recognized enterotoxin. Work is under way to characterize a putative new EPEC enterotoxin.

Volunteers immunized with two parenteral doses of the Brinton *E. coli* purified type I somatic pilus vaccine were not significantly protected against oral challenge with a virulent strain of *E. coli*. This challenge strain had a type I somatic pilus closely related to, but not identical with the vaccine pilus. An efficacy study of this type I pilus vaccine against an *E. coli* strain bearing a serologically identical type I pilus type is planned.

A laboratory derived mutant Ogawa strain of *V. cholerae*, Texas Star-SR (toxin A^- B^+) was fed safely as a vaccine to volunteers in one large dose. Seven weeks later, a virulent Ogawa El Tor strain produced diarrhea in 7/10 controls whereas only 1/7 Texas Star-SR vaccinees developed diarrhea. The vaccineees shed 1000-fold fewer virulent organisms than did the controls, suggesting that Texas Star-SR induced antibacterial immunologic protection. These results are encouraging with respect to the possibility of developing a safe and effective live attenuated bacterial vaccine for cholera.

An oral, attenuated rotavirus type II vaccine was fed safely to two seropositive volunteers. Ten additional volunteers with low, serum neutralizing rotavirus titers fed this candidate vaccine did not develop diarrhea. Clinical laboratory tests are pending, and if normal, the vaccine will be tested in additional adult seronegative volunteers.

A cold-adapted, attenuated influenza vaccine, A/California/10/78 (H_1N_1), administered intranasally, was well tolerated and immunogenic.

Collaborative Clinical Trials of Antibiotic Therapy

To stimulate clinical trials of antibiotics, a program announcement entitled "Controlled Clinical trials for Prophylaxis or Improved Antimicrobial Therapy of Selected Bacterial and Mycotic Infections" was published during the first quarter of FY '81. There has been a good response to the program announcement. Three applications were received in February, 1981, and six applications at the second receipt date on June 1, 1981.

The contract with the University of Alabama to study improved therapy of cryptococcal meningitis and other systemic mycotic diseases has provided significant clinical and laboratory data during its third year of operation. The present study design requires a total of 92 randomized patients. Accordingly at least 35 additional patients will be enrolled during the next 18 months of the contract. Ongoing studies include Study #1 which is a comparison of two different regimens of 5 fluorocytosine (5-FC) and Amphotericin B (AMB) in the therapy of cryptococcal meningitis. This study, which compares a 4 weeks regimen of 5-FC and AMB with a 6-weeks regimen using the same drugs at the same dosage, is designed to determine the most efficacious, minimum duration regimen for cryptococcal meningitis. Renal transplant recipients have been identified as a group of high risk patients who clearly should be treated for the full 6 weeks.

Serotyping studies of C. neoformans isolates have been performed on ninety clinical isolates. The results suggest that BC serotypes, previously thought to be strictly limited in their geographic occurrences to southern California and southeast Asia, may have a greater distribution than originally thought. The epidemiologic significance of this observed BC distribution, as well as the success of treatment in serotype AD versus serotype BC infections, are being evaluated. Stored sera and cerebrospinal fluid from groups of study patients are being tested for cryptococcal antigen to determine the value of serial cryptococcal antigen titers in predicting outcome and duration of therapy.

During the past year, Study #2--"Phase II Studies on Ketoconazole (Keto)" was completed. This study was designed primarily to obtain in vitro and toxicity data on Keto, an investigational oral imidazole agent with promise of less toxicity then either 5-FC or AMB. Based on the excellent in vitro antifungal activity of Keto and the lack of serious toxicity on the bone marrow, kidney and liver observed in Study #2, the following phase III studies with Keto were initiated: Study #3A--a comparison of a low dose Keto regimen (400 mg/day) versus a high dose Keto regimen (800 mg/day) to provide information on the optimal dosage and duration of therapy for patients with blastomycosis or histoplasmosis. A second phase III study--#3B was initiated in April, 1981. This trial compares the efficacy and toxicity of a low dose (400 mg/day) versus a high dose (800 mg/day) regimen of Keto in randomly assigned patients with non-meningeal coccidioidomycosis. The purpose of this study is to determine the best dosage and duration of Keto therapy of this systemic mycotic disease which is sometimes refractory to conventional chemotherapy with Amphotericin B.

During FY '81 program staff decided to terminate the contract with the University of Illinois by July, 1982. This contract was a clinical trial designed to perform a placebo-controlled, double blind evaluation of the efficacy of penicillin in the prevention of early onset Group B Streptococcal (GBS) sepsis in the newborn. A variety of adverse circumstances prevented the contractor from enrolling sufficient mother-infant pairs into the trial during the first two year award period. Actually, 2500 infants were enrolled--far short of the contractor's initial estimated enrollment of 10,000 over a three year period. Although the trial itself was not completed, sufficient data have been collected to evaluate risk factors and other epidemiologic features of the disease. Reaction to penicillin injections occurring in the first days of life have been carefully recorded. So far, with over 2700 injections, no major reactions occurred. Non-specific rashes and local swelling occurred with equal frequency in infants receiving penicillin or placebo.

Nutrition, Infection And Immunity

The goal of this program is to promote research on the interaction of malnutrition, infection and immunity. Specifically, our interests focus on the modulating effect of specific nutrients on immune function and microbial virulence, on mechanisms of food allergies and immune response to ingested antigens, and on the interaction of nutrition and infection in the tropical environment and in American hospitals.

In FY '81 the NIAID Program in Nutrition, Infection and Immunity consisted of 22 grants, small portions of two contracts, one New Investigator Award, and a portion of one training grant. One intramural research project on Food Allergy was continued, and a new RFA on Food Allergy was advertised. The extramural program was monitored by at least four project officers in the IAID and MID programs, in addition to the CESB.

During FY '81, Dr. Edelman resigned his appointment as Vice-Chairman of the NIH Nutrition Coordinating Committee (NCC), but continued as Institute representative to the NCC. He served as *ad hoc* consultant to the NCI on several of their contracts on Nutrition and Cancer, and chaired an NCC group drafting a trans-NIH Program Announcement on "Support of the Patient by Means of Enteral and Parenteral Nutrition." Dr. Edelman was appointed to the Editorial Boards of *Nutrition Research* and the *American Journal of Clinical Nutrition*.

Rapid Viral Diagnosis

Currently there are three grantees and three contractors in the RVD program. Grantees are attempting to devise tests for the early diagnosis of LaCrosse arboviral encephalitis, respiratory virus infections, and group B coxsackievirus infections. The contractors are studying the enzyme-linked immunosorbant assay (ELISA) and modifications of it, as described below.

Dr. McIntosh at Harvard has continued work on an ELISA for respiratory syncytial virus using commercially available reagents. Comparing three diagnostic tests for RSV, the ELISA was somewhat less sensitive than immunofluorescence and tissue culture for virus detection.

Dr. Richman at the University of California, San Diego, has continued work on his staphylococcal protein A/radioimmunoassay (SPA/RIA) characterizing its sensitivity and specificity and applying it to new viruses. He has adapted it for the rapid detection of serum antibody against varicella-zoster virus, and to the identification of HSV types 1 and 2, VZV and CMV antigens, using as little as a single infected tube culture. Using a battery of selected monoclonal influenza antibodies, he can identify and distinguish recently circulating variants of influenza A and B in a single tube culture. In collaboration with investigators at the Centers for Disease Control (CDC), he has adapted his system to measure antibodies and antigens of arenaviruses and the Ebola-Marburg viruses, and interesting epidemiological observations have been made using African sera.

Dr. Yolken at Johns Hopkins has discovered that complement can amplify the enzyme immunoassay systems about 10-fold for the detection of rotavirus and Hemophilus influenzae antigens, without loss of specificity. He has developed a 30 minute assay for the measurement of H. flu antigen in the cerebrospinal fluid of meningitis patients, and will apply this rapid ELISA technique to other pathogens. Dr. Yolken has developed a cytomegalovirus specific IgM antibody test for the rapid diagnosis of congenital CMV infections. This test was capable of detecting IgM antibody in all of 10 babies with congenital CMV infections, but yielded no false-positives in 66 healthy babies, a considerable improvement over current serological tests. Dr. Yolken has developed an ELISA assay for more than 30 serotypes of human adenovirus, using a shared internal hexon antigen. The adenovirus so identified is then serotyped by differential binding or blocking assays. With this assay he has identified non-cultivatable adenovirus type 38 in 12 of 96 stool specimens obtained from children with diarrhea not caused by any known pathogen. The stool of 220 healthy infants were negative for adenovirus 38. In addition, he has found that hospital outbreaks of combined diarrhea and respiratory disease occurring during the Fall were associated with adenovirus type 38 infections. Dr. Yolken has also provided direct evidence that selected viruses are associated with acute otitis media. From middle ear fluids of 53 children presenting with acute otitis media, S. pneumoniae was found in 19, RSV in 10, H. influenzae in two, and rotavirus in one. Together with conventional serological evidence, the ELISA results support the conclusion that RSV plays an important role in the pathogenesis of otitis, especially when otitis occurs during the period when RSV is prevalent.

A workshop titled "New and Useful Techniques in Rapid Viral Diagnosis" was co-sponsored by this program last year. A summary of it was published in the Journal of Infectious Diseases 142:793-802, 1980.

Epidemiology and Biometry Section

With the departure of Dr. George Curlin as Branch Chief early in the year, the branch was reorganized into the Epidemiology and Biometry Section (EBS) with Dr. Richard A. Kaslow as Section Head. The reorganization of the unit led to a reallocation in staff time and other resources. While continuing to service the small epidemiology grant/contract portfolio, a commitment was increasingly made to direct internal research activities. After a year of academic training, Dr. Alfred Saah joined the Section as an Epidemiology Training Associate.

Consultation and Biometric Research

Dr. William Blackwelder served as consultant to NIAID project officers and as a member of data monitoring committees for collaborative clinical trials on: 1) comparison of two durations of therapy with amphotericin B and 5-fluorocytosine in cryptococcal meningitis, 2) efficacy of prophylactic penicillin for group B *Streptococcus* infections in neonates, 3) efficacy of inactivated hepatitis B vaccine in patients and staff of dialysis units, and 4) efficacy of adenine arabinoside monophosphate alone and combined with human leukocyte interferon in chronic hepatitis B infection. Dr. Blackwelder continued to collaborate with Dr. David Alling, OSD, NIAID, and Dr. Sir Charles Stuart-Harris, Fogarty Scholar, in a comparative study of the effect of influenza activity on mortality from chronic obstructive lung disease in Great Britain and the U.S. He also continued to work with Dr. John LaMontagne, DAB, and Mrs. Jacqueline Smith in data analysis for a study of neurological symptoms reported in the 1978 Russian influenza vaccine trials. Dr. Blackwelder collaborated in data analysis for a study to establish a U.S. reference for human serum immunoglobulin E. He also collaborated with NHLBI contract researchers in presenting a comparison of methods for diagnosing angina pectoris in the Honolulu Heart Study. Dr. Blackwelder elaborated on the appropriate statistical hypothesis for comparing a standard to an equally effective new regimen, and carried on earlier work with Dr. Robert Elston of Louisiana State University on statistical properties of tests for genetic linkage using sibship data. Manuscripts are in various stages of preparation for many of these studies.

Mrs. Smith provided computation assistance and consultation for the Washington, D.C., Children's Hospital contract research on diarrhea disease, and continued to exploit existing health surveys that provide data on the cost of illnesses of concern to the Institute. Dr. Kaslow and she also began developing a data base from 1968-70 national mortality statistics. As that data base evolves, attempts will be made to use this source to examine infection as a consequence of diabetes, cancer, etc., and to explore conditions associated with immunologic diseases such as asthma, insulin-dependent diabetes, and rheumatoid arthritis. Mrs. Smith left the Institute late in the fiscal year for a position in another Institute.

Infectious Diseases

Considerable attention was given to the role of histocompatibility antigens (HLA). Dr. Blackwelder and two former NIAID staff members completed the analysis of a study of HLA and occurrence of herpes virus infections. Dr. Kaslow completed two reviews in the area, one on the epidemiology of reactive arthritis and the other on HLA and infection in general; both are in press. The field work on HLA and immune response to vaccines under contract with Johns Hopkins (Dr. Bias) has proceeded more slowly than expected, so results of the laboratory work, although promising, are not yet available. Plans for study of HLA in tuberculosis have been formulated, and limited work on identifying specific agents of Reiter's syndrome continued.

Two collaborative projects have been conducted. Drs. Blackwelder and Kaslow have assisted Dr. David Henderson and Ms. Joan Laniak, OAM, CC, in a study on mediastinitis in patients undergoing cardiac surgery in the Clinical Center. Drs. Saah, Kaslow, and Curlin have worked with Dr. LaMontagne, DAB, in performing a retrospective assessment of "apparent" efficacy of influenza vaccine among residents of the Jewish Home and Hospital for the Aged in New York City. Preliminary results indicate that patients requiring skilled nursing who developed pneumonia during the past two influenza seasons were roughly half as often vaccinated as not vaccinated. Further data from the retrospective study will be analyzed, and plans involving virologic studies are under way for prospective evaluation during the next influenza season.

Immunologic Diseases

The case-control study on HLA in black systemic lupus erythematosis patients at Johns Hopkins Hospital was undertaken, and the questionnaire on risk factors for that condition began to be pilot tested.

The unit was involved in two projects on asthma. Drs. Kaslow and Daniel Mullally, IADP, reviewed samples of records on children admitted to Children's Hospital Medical Center for asthma. Demographic changes in the asthmatics being cared for appeared to account in part, but not entirely, for the increase in numbers of admissions for asthma over the previous 20 years. In addition, Drs. Saah and Kaslow have made preliminary arrangements to analyze data from the Perinatal Collaborative Study in an attempt to identify maternal factors during the prenatal period and early postnatal factors related to the occurrence of asthma in the cohort of children included in this survey.

Other Activities

Mrs. Mattheis collects and reviews the necessary documentation required to file and maintain Investigational New Drug Applications (INDAs) with the Food and Drug Administration (FDA) for the Microbiology and Infectious Diseases Program. Currently, nineteen active INDAs are filed with the Bureau of Biologics, FDA, and seven with the Bureau of Drugs, FDA. During the past year the sponsorship of an IND for the "Capsular Polysaccharides of Types Ia, Ib, II and III Group B *Streptococcus*" was transferred from Harvard to the NIAID, and new INDAs were filed for "Live Human Rotavirus (Wa strain, subgroup 2) vaccine candidate," and "Human Leukocyte Interferon and Adenine Arabinoside Monophosphate."

The branch staff participated as members of many trans-NIH committees. Dr. Edelman and Mrs. Smith represented the Institute on the Digestive Disease Coordinating Committee. Dr. Kaslow and then Dr. Horton functioned as the Institute's representative to the NIH and PHS Diabetes Committees. Dr. Kaslow was active in the NIH Epidemiology Committee, and served as a resource consultant to the Diabetes Coordinating Committee. Dr. Horton served on the Cystic Fibrosis Coordinating Committee, and Dr. Edelman served on the NIH Nutrition Coordinating Committee and the NIH Clinical Trials Committee. Dr. Edelman continued to serve on the following committees of other Federal agencies: the Human Ethics Committees (Institutional Review Boards) of the Walter Reed Army Institute of Research and the Frederick Cancer Research Center. As Clinical Associate Professor of Medicine, Dr. Edelman served as a preceptor in Clinical Medicine, Uniform Services University of the Health Sciences.

DEVELOPMENT AND APPLICATIONS BRANCH

Consistent with its name, the primary objective of the Development and Applications Branch is the translation of new information derived from basic research into methodologies appropriate for the control or prevention of infectious disease areas designated as high priority for extramural support by the Institute. Fundamental and applied research activities include: identification of important infectious disease problems with potential for control or prevention through immunization or utilization of antivirals or hyperimmune sera, development of appropriate vaccines, antivirals, and other control measures, design and support of appropriate clinical trials for evaluation of control measures and basic research in related areas.

Approximate Level of Support

Activity		Number	Amount
Vaccine Evaluation Units			
Research Contracts		5	$ 889,758
	Subtotal	5	$ 889,758
Influenza			
Research Contracts		9	1,347,514
Interagency Agreements		1	85,000
Research Grants		24	2,508,141
Young Investigator Awards		3	139,501
Career Awards		1	53,721
	Subtotal	38	$ 4,133,877
Respiratory Diseases			
Research Grants		6	480,503
	Subtotal	6	$ 480,503
Hepatitis			
Research Contracts		6	1,477,346
Interagency Agreements		2	175,479
Research Grants		8	1,109,245
Young Investigator Awards		1	40,403
Fellowships		2	38,080
	Subtotal	19	2,840,553
Antiviral Substances			
Research Contracts		15	1,351,222
Research Grants		36	3,126,679
Career Awards		2	79,000

Approximate Level of Support (cont.)

Activity	Number	Amount
Antiviral Substances (cont.)		
Fellowships	3	57,816
Training Grants	1	90,478
Subtotal	57	$ 4,705,195
Bacterial Vaccines		
Research Contracts	11	598,090
Research Grants	27	2,330,570
Young Investigator Awards	1	44,467
Career Awards	2	88,951
Fellowships	6	105,656
Subtotal	47	$ 3,167,734
Viral Vaccines		
Research Contracts	1	0*
Research Grants	7	618,158
Young Investigator Awards	1	34,434
Fellowships	1	19,040
Subtotal	10	$ 671,632

*Prior year funds

Branch Summary

Activity	Number	Amount
Research Contracts	47	$ 5,663,930
Resarch Grants	108	10,173,296
Interagency Agreements	3	260,479
Young Investigator Awards	6	258,805
Fellowships	12	220,592
Career Awards	5	221,672
Training Grants	1	90,478
Totals	182	$16,889,252

Program Summary

Comprehensive research programs on influenza, other viral respiratory diseases, viral hepatitis, viral vaccines, bacterial vaccines and antiviral substance development are components of the Development and Applications Branch. Because these programs are designated as high priority for development of control measures, funding is provided through all mechanisms available to staff. The DAB accomplishes most of its activities through directed research, and is thus responsible for the major portion of contract activities within the Microbiology and Infectious Diseases Program.

In spite of approximately 20 percent reduction of contract funds within the Institute, we have been able to continue research activities that are contributing new and significant information towards our goals of disease control in the areas of responsibility. However, program vigor is dependent, in large part, on new activities and follow-up of promising research. In view of current estimates for future years' support, we must look upon our activities as "bare-bone." The planning for allocation of research dollars is extensive and detailed, and we are firmly convinced that the activities being supported represent essential basic components of the various programs and that individual contractors represent the best expertise available in the country. Further reductions in resources will greatly decrease our chances for obtaining "payoffs" in the future.

Influenza viruses continue to produce surprises for investigators. The epidemic of 1980 was due to a combination of H3N2 and H1N1 viruses, and caused excess mortality throughout the U.S. Disease and mortality from influenza viruses were not restricted to man. A large kill of seals off the New England coast has been attributed to influenza infection. Infections in swine have reappeared in Europe this year with attendant disease. Recombinant DNA technology is contributing heavily to basic research on viral polypeptides and nucleic acids. Definitive studies on rimantadine and amantadine as prophylactic agents for influenza virus were obtained this year. It was shown that rimantadine was as effective as amantadine, but virtually free of the bothersome side effects frequently associated with amantadine. Cold adapted viruses remain viable candidates for attenuated influenza vaccines, but much research remains to be performed.

The hepatitis program has had a rewarding year. Hepatitis B immune globulin was shown to be effective in preventing the chronic carrier state in babies born of carrier mothers. Hepatitis B vaccine has been shown to be safe, highly immunogenic, and efficacious (in male homosexuals). Efficacy trials in hemodialysis patients and staff are well under way. These two advances in control of HBV infections must be tempered by the fact that the regimens are expensive. Alternate sources of antigen for vaccine are being pursued through the use of DNA recombinant technology and/or chemical biosynthesis. During the past year woodchucks were successfully bred and raised in captivity, offering the prospects of laboratory animals for studies on viral induced primary hepatocellular carcinoma. Prospects for an attenuated hepatitis A vaccine look encouraging.

Interferon continues to receive large amounts of publicity because of its potential as an antitumor agent. While hopes for a "miracle drug" have faded, there is general agreement that interferon will have a place in treatment of cancer and viral infections. The DAB is closely following developments with cloned interferon subtypes produced by recombinant DNA techniques, but current clinical trials will be performed using native cell-produced species until more data are available on the activities of the cloned subtypes. The most exciting data on antiviral drug therapy during the past year is that adenine arabinoside is effective in treatment of herpes zoster infections in the immunosuppressed. This, coupled with data accumulating on acyclovir, represents a major step forward in treatment of serious herpesvirus infections.

The Bacterial Vaccines Program has been successfully coordinating trials in various "at risk" populations for immune responses to pneumococcal vaccine.

Most patient groups appear to mount normal antibody response to the vaccine. Group B meningococcal polysaccharide is non-immunogenic in man, but a preparation consisting of a complex of outer membrane proteins and B polysaccharide is immunogenic in adults and adolescents, and may be a vaccine candidate for children and infants. These studies are planned for the forthcoming year. Epidemiologic studies on Rocky Mountain Spotted Fever are continuing, and more precise data should be available in the near future on disease prevalence and incidence.

The primary agents of lower respiratory disease in pediatric populations, i.e., RSV and parainfluenza viruses, are being biochemically and biophysically characterized. Epidemiologic studies are contributing important new insights on the natural history of RSV infections.

In FY81 the DAB was involved in the organization and conduct of three meetings or workshops: the Second U.S.-Japan Joint Hepatitis Panel Meeting in Hakone, Japan; Acyclovir Workshop; and Genital Herpes Antiviral Agent Workshop (both held in Bethesda). The DAB co-sponsored two additional meetings - Genital Herpes Vaccine Workshop and the Reye's Syndrome Consensus Development. Also, two major meetings, an International Hepatitis Symposium in New York City and the International Conference on the Biology of the Interferon System in Rotterdam had program relevance and support. A DAB staff member served as co-chairman of the latter meeting.

Research Highlights

Influenza Program

This last year was once again a year of significant activity in influenza. The impact of influenza virus infections on the health of the U.S. population was particularly noteworthy. In fact, the Centers for Disease Control estimated that the 1980-1981 epidemic due to the A/Bangkok/79 (H3N2) and A/Brazil/78 (H1N1) viruses resulted in 60,000 to 70,000 excess deaths. Excess mortality was recorded for 13 consecutive weeks and was widespread throughout the United States. The last epidemic of such magnitude occurred in 1968-1969 when A/Hong Kong/68 (H3N2) viruses first appeared. The circulation of two antigenic types has also continued. This situation is perplexing since it casts doubt upon the concept that new variants quickly displace older viruses and become the predominant virus. Although the H1N1 viruses reappeared in 1977 they have not succeeded in replacing the older H3N2 viruses. The occurrence of recombinant viruses between the H3N2 and H1N1 viruses is also perplexing. Recombinants were found between the A/Brazil/78 (H1N1) viruses and A/Texas/77 (H3N2) viruses during the 1978-1979 epidemic. While these recombinant viruses were the most common type identified during that winter they did not reappear last year to any significant extent. Both H3N2 and H1N1 viruses occurred, but recombinant viruses were infrequently found.

The significance of influenza viruses as animal pathogens was also particularly important during the last year. A virulent outbreak of pneumonia among harbor seals was reported last year with hundreds of dead seals being washed ashore along the New England coast from January to May, 1980. The virus responsible, A/Seal/Massachusetts/8/80 (H7N7), was recovered in high titers from autopsy specimens of the lungs and brains of affected seals. The virus was found to be antigenically and genetically related to avian myxoviruses, and a similar virus was isolated from migratory birds in Iceland. The A/Seal/Massachusetts/8/80 (H7N7) virus was found to contain genes of avian origin, yet it exhibited a marked tropism toward mammalian tissues. It can infect ferrets, for example, and has been found to produce conjunctivitis in humans. The significance of this virus as a possible pathogen for man is unknown, but certainly continued attention will be focused on it. In addition, swine influenza viruses have reappeared in Europe and have produced significant disease in pigs this year. There have been no reports of human infections.

One swine virus, A/Mayo Clinic/74 (Hsw1N1), isolated from a patient in 1974, was recently found to be a recombinant virus. This virus apparently contains the H3N2 genes for the P1, P2, P3 and NP proteins. These genes were found to be nearly identical to the corresponding genes from the A/Port Chalmers/74 (H3N2) virus. This gene constellation is the same as that found in the 1978 H1N1-H3N2 recombinants.

Important new information has been obtained by NIAID-supported investigators regarding the basic properties of influenza viruses. The three dimensional structure of the influenza hemagglutinin has been solved. This information has given new insights into the process of antigen variation. The possibility of producing inactivated vaccines that contain only the necessary antigenic determinants is being actively investigated. The role of endogenous mRNA in the synthesis of viral messages has been a hotly pursued research objective. Extramural and intramural scientists have clearly shown that the capped 5' end of cellular mRNAs are transferred to viral mRNAs. Intramural scientists, in collaboration with NIAID grantees, have shown that this process is mediated by viral proteins. None of this work would have been possible without the application of recombinant DNA technology to these problems. This methodology has been applied to many of the recent studies of influenza virus replication. It was instrumental in the identification of two additional viral mRNAs, NS2 and M2. Apparently, then, the eight genes produce at least 10 proteins. These techniques have also increased the available information on the structure of the other genes. Recent developments include the expression of influenza virus HA gene cloned in an SV40 vector in African green monkey kidney cells and cloned in PBR 322 in *E. coli* cells. Large amounts of hemagglutinin may soon be available for other studies.

The pursuit of improved methods of prevention and/or treatment has been given high priority. A recent study conducted by the University of Vermont has conclusively shown that rimantadine is as effective as amantadine in the prevention of influenza infections. Moreover, rimantadine was found to have virtually no neurological side effects. Therapy of influenza infections has also been an area of active research. Amantadine has been shown to be effective when used therapeutically in cases of uncomplicated influenza infections in normal young aults. Recent efforts have focused on an assessment of amantadine's therapeutic potential in severe influenza infections. In addition, this antiviral compound has been compared to other drugs which have been traditionally used for influenza infections. Side effects associated with aspirin and chlortrimeton were shown to occur with greater frequency than side effects observed in amantadine-treated patients. Research into improvements in the laboratory diagnosis of influenza infections has been successful. A sensitive ELISA method for IgG and IgM antibodies was developed. Methods for the rapid identification of virus in nasal secretions are being tested, and new cell substrates for the direct isolation and propagation of influenza A viruses have emerged.

A second area of high priority is the development of an attenuated vaccine for influenza A. Recent clinical and laboratory studies have shown that the cold-adapted vaccines are antigenic, avirulent and genetically stable. Additional studies need to be conducted before this can be confirmed, but the data with these vaccines are very encouraging. The ability of these vaccines to protect individuals from natural challenge needs to be further evaluated, and will be the primary focus of clinical trials for the next several years.

Reye's Syndrome is a major concern of the influenza program. This serious disease is associated with influenza virus epidemics. Recent data suggest that the use of aspirin to treat prodromal respiratory illness may significantly increase the risk of Reye's Syndrome. This disease was also the focus of a consensus development conference in March of this year. The conference, co-sponsored by the NIAID, NINCDS, NICHD, NIEHS and NIADDK, was quite successful, and will be an important landmark in the efforts to control Reye's Syndrome. The conference established standards for diagnosis, staging of coma, and treatment. Additional research will be needed on etiology, risk factors, diagnosis, and treatment before the disease is controlled. Towards this end, the NIAID co-sponsored a program announcement on Reye's Syndrome, and research grant applications in response to that announcement are now in the review process. It is hoped that more investigators will be attracted to this important area of research in the future.

Viral Respiratory Diseases Program

The magnitude of infections and illnesses from parainfluenza viruses types 1, 2, and 3, and respiratory syncytial virus (RSV) is difficult to document, but it is estimated that 17 percent of pediatric infections are due to these agents. Of all severe lower respiratory diseases in infants and young children, perhaps 40 percent could be prevented if effective vaccines against these agents were available and used.

Successful immunization against these agents represents a formidable task. The immune state following natural infection, if existent, is short-lived and does not correlate with serum neutralizing antibody. RSV represents a serious threat to infants because the primary infection is associated with increased risk of lower respiratory disease. Efforts to develop attenuated RSV vaccines that will mimic natural infection, but not induce illness, have hit snags during the past year. Administration of ts-2 to seven seronegative babies resulted in only two infections. No illnesses were observed. Thus, all data collected on ts-2 indicate that it is over attenuated and can no longer be considered a vaccine candidate. Other mutants (NG-1 and NG-16) lost many of their attenuation characteristics after propagation in human diploid cell cultures (MRC-5). Therefore, no promising live RSV vaccine candidates are currently available for evaluation.

Elucidation of the role of fusion protein (F) in paramyxovirus spread in tissue culture systems has provided new impetus to identifying such a component in parainfluenza 3 and RSV. The MIDP has released an RFP for research on fusion glycoprotein of parainfluenza 3. Studies to be performed include identification of fusion protein; development of serologic assays for F protein and anti-F; sensitivity of F protein to routinely used vaccine inactivants (formalin, tween); isolation and purification of various surface glycoproteins; and evaluation of vaccine potential either as subunit or whole virus preparation in hamsters. If successful, a small amount of vaccine will be prepared for human evaluation.

Similar studies are being undertaken with RSV by the Molecular Virology and Immunology Laboratory, Georgetown University. This is a difficult agent to work with because of low virus yield from cell cultures and because of virus instability. The Georgetown Laboratory has described the number and sizes of the RSV constituent polypeptides and glycoproteins, identified the RNP, developed a double antibody radioimmunoassay for anti-RNP, prepared specific antisera to RNP and the "spike" glycoproteins, identified a phosphoprotein and protein kinase activity in purified virions, and described a 47S RNA associated with the RNP from infected cells and released virus. Progress during the past year includes: development of a method for stabilizing the virus during purification; development of Balb/c cells persistently infected with RS virus and the expression of RNP on its surface; and the production of monoclonal antibodies to both the coat and RNP proteins of the virus. Work is continuing with the immediate goal of the functional assignment of the structural virus-specific proteins and glycoproteins.

Studies on the natural history of RSV infections and risk factors associated with lower respiratory disease are being conducted at several vaccine evaluation centers. Young, healthy adults who had previous infections with RSV were susceptible to reinfection -- many of whom developed febrile respiratory illness. Volunteers who acquired RSV infection naturally in 1979 were challenged with an RSV infectious pool. Virus was given two months after natural infection, then at four months, eight months, fourteen months, and twenty months. The highest proportion of volunteers became infected on the first challenge at two months. It appeared that those volunteers who did not become infected were those, with one exception, who had had the most symptomatic natural illness.

Children with congenital heart disease and those immunosuppressed are at increased risk for severe infections or death from the major viral pathogens such as RSV, influenza, and parainfluenza viruses. It appears prudent to avoid elective admissions of infants with congenital heart disease during the period that RSV is prevalent in the community. If admitted, nosocomial infection should be avoided by protective handwashing by staff, and protective isolation, if feasible, should be utilized.

When a specific antiviral agent(s) and/or efficacious vaccine is available, infants with congenital heart disease, and especially those with associated pulmonary hypertension, could be prime candidates for therapy. This, plus prudent infection control measures, may play a significant role in decreasing mortality in these infants.

The paucity of candidate vaccines for the respiratory viruses is a reflection of the difficulties in working with the viruses, lack of understanding of mechanisms of pathogenesis and immunity, and no readily available animal models. However, the DAB is prepared to evaluate potential candidates developed within industry and academia, as well as Government laboratories.

Viral Hepatitis Program

The goal of the Hepatitis Program is development of control measures for hepatitis A (HAV), B (HBV), and non A-non B (NANB) infections. Because of its more serious clinical manifestations and long term sequelae, HBV is of major public health importance in the U.S. It has been estimated by the Centers for Disease Control that 200,000 HBV infections occur each year in the U.S., with resultant costs of approximately $750,000,000. Of those infected, about 2.4 percent will die from cirrhosis or primary hepatocellular carcinoma, diseases closely associated with long-term chronic infection. Moreover, the global impact of the disease has even greater significance because it has been estimated that 176,000,000 carriers serve as a reservoir of infection.

During the past year, data were analyzed from the hepatitis B immunoglobulin trial being conducted in Taiwan. HBV infection early in life often leads to the HBsAg carrier state with its inherent risk of chronic hepatitis, cirrhosis, and primary liver carcinoma. Babies born to HBsAg+ HBeAg+ carrier mothers, were randomized into three treatment regimens: placebo, HBIG given at birth, three and six months, and HBIG administered only once, shortly after birth. Follow-up visits were scheduled for every three months until the babies were 24 months of age. Two hundred and eighty-two babies were entered into the study, with seventy-three having completed one year or more of follow-up. Overall rates of HBV infection did not differ between the three study groups. However, two important differences in the course of infection were observed. The age of the infant when infection was first detected was later in HBIG treated infants. More importantly, although the rate of infection was not influenced by HBIG treatment, the frequency of chronic antigenemia was markedly reduced. Of 24 infants given the placebo, 92 percent had persistent infections. In contrast, 46 percent of 28 infants given a single HBIG injection at birth and only 19 percent of 21 infants treated with the three dose regimen became chronic carriers. These data unequivocally demonstrate the efficacy of HBIG treatment in preventing the chronic carrier state in infants of HBsAg carrier mothers.

Data that show HBV vaccines consisting of formalin inactivated 22 nm spheres of HBsAg absorbed to alum are safe, immunogenic, and efficacious continue to accumulate. The vaccine developed by the NIAID has been administered to over 275 adult volunteers. Side effects were negligible, and seroconversion rates were impressive, with at least 28 percent of the vaccinees developing anti-HBs by two weeks and 65 percent within one month after the first dose of vaccine. Overall, 98.5 percent of the study group developed anti-HBs after three doses of vaccine. Based on the geometric mean anti-HBs response, observed three months after the last injection, the highest geometric mean anti-HBs response occurred in the group receiving 40 µg HBsAg at 0, 1 and 6 months. However, this was not significantly different from the responses observed in participants who received a second injection at two months and a third at four or six months. There was no difference in the frequency of seroconversions to 10, 20, or 40 µg of antigen. However, the geometric mean antibody titer was higher in those receiving 40 µg. The NIAID vaccine is currently being evaluated for reactogenicity and immunogenicity in normal children, ages 15 years down to 1 year.

The NIAID-supported trial being conducted in hemodialysis patients and staff is well under way. The trial, employing Merck Sharpe and Dohme adw vaccine, involves 45 hemodialysis centers through the U.S. Randomization into the double-blind study ceased on March 1, 1981; 1,336 patients and 889 staff members have been enrolled. Attack rates in the dialysis units have been steadily decreasing over the past two years, and HBV events are occurring very slowly. It appears clear at this point that efficacy determination in staff will not be feasible. However, safety and immunogenicity of the vaccine have been firmly established. It is hoped that the number of events occurring in the patient population will permit a determination of efficacy within the next year. It is likely that an additional one year of follow-up will be required for efficacy assessment, but funds are not available for continuation past May 30, 1982. Based on data described last year and additional data accumulated during this year, Merck Sharpe and Dohme has made application to the BoB/FDA for licensure of their vaccine. Disposition of the application has not yet been made.

Simultaneous administration of HBIG and HBV vaccine in adults has been shown to confer significant levels of anti-HBs from time of immunization. The HBIG does not suppress antibody formation to the HBsAg. This observation will be highly significant in developing trials and strategies for prevention of neonatal infections and other post-exposure events. The NIAID and CDC have entered into an agreement with the People's Republic of China (PRC) for evaluation of HBV vaccine in preventing maternal-fetal transmission of HBV. The vaccines to be tested will be from the NIAID and PRC.

Current HBV vaccines depend on plasma from chronic carriers and represent "first generation" vaccines. Although they are safe, immunogenic, and efficacious, it is anticipated that expense will be a deterrent to their widespread utilization. Recent successes in developing alternate sources of antigen through recombinant DNA technology and/or biochemical synthesis of individual polypeptides promise to contribute to second and third generation vaccines that will be equally immunogenic and efficacious and cost substantially less.

Dr. Gerin, an NIAID contractor, and colleagues have successfully incorporated the nucleotide sequences for HBsAg into the animal virus SV40 and then transfected African green monkey kidney cell cultures. The antigen that was secreted into the culture medium was identical to that found in sera of patients with type B hepatitis. Purification and inactivation of the cell-produced antigen resulted in a preparation that was highly immunogenic when inoculated into chimpanzees. The immunized chimpanzees have been challenged with wild-type virus to test efficacy.

The treatment of patients with chronic hepatitis B using human leukocyte interferon and/or adenine arabinoside has been studied at Stanford University by Merigan, Robinson and colleagues. Their results appear to be encouraging and controlled studies are in progress. (See Antivirals Section)

The development of the woodchuck (Marmota monax) as a model for virus-caused hepatoma has progressed. Animals were bred in captivity and a number of litters were born and raised, indicating that woodchuck pups can be realistically produced in captivity. The immediate problem is that the pups will probably have to be infected immediately after birth or be born of chronic carrier mothers to develop the chronic carrier state. This necessitates that the contractor also be capable of providing a separate and isolated experimental facility. Assays for WHsAg, anti-WHs, WHeAg, anti-WHe, and DNA polymerase have been developed and are specific and sensitive. As reported last year, NIAID grantees are investigating the ground squirrel (Spermophilus beecheyi) as a possible model of disease induced by a virus similar to human HBV.

The chimpanzee breeding colony which is jointly supported by the NIAID, BoB/ FDA, and CDC is now under the care of the New Mexico State University. The personnel on site at the colony were transferred from Albany Medical College to NMSU. The transition progressed smoothly and the colony appears to be managed quite successfully. The close proximity of NMSU to the colony itself will be greatly beneficial to the sound management of this valuable resource.

The breeding colony for Saguinus mystax marmosets at the Delta Regional Primate Center (DRPC) was terminated during the past year. The requirements for Saguinus marmosets, however, remain unchanged. The BoB/FDA, CDC, and NIAID have entered into an agreement with the Division of Research Services for re-investigation of marmoset breeding. A colony of S. mystax and S. labiatus is being established at the Poolesville Animal Farms. Multiple factors, such as diet, housing, and hormonal balances, will be studied. To date, these marmosets have not been successfully bred in captivity.

The report of the successful cultivation of hepatitis A (HAV) in cell culture by Provost and Hilleman has been confirmed in a number of laboratories. HAV has now been propagated in a variety of cell cultures, including primary explant cultures of marmoset liver, fetal rhesus kidney cells, primary African green monkey kidney cells, various hepatoma cell lines, and more recently in a human diploid cell line. No cytopathology has been observed, but the virus has been identified by immunofluorescence, serum neutralization, radioimmunosassay, immune electronmicroscopy, and marmoset inoculation tests. Merck Sharpe and Dohme has shown that formalin inactivated HAV is non-infectious and protects against challenge with wild type virus in marmosets, thus establishing the principle of an inactivated HAV vaccine. Investigators at the NIAID report that HAV serially passaged in African green monkey kidney cells appears to be losing virulence for chimpanzees, suggesting possibilities for a live attenuated vaccine. Much remains to be done with in vitro cultivation of HAV, however, before sufficient antigen becomes available for vaccine use.

Extensive studies of non A-non B (NANB) hepatitis by various investigators have shown that at least 90 percent of cases of post-transfusion hepatitis are caused by NANB agents. Two studies have revealed a significant correlation between the level of serine alanine aminotransferase (ALT or SGPT) in donor blood and subsequent development of NANB post-transfusion hepatitis. In view of these findings, it is likely that donor blood will be tested for the presence of elevated ALT levels. Although NANB hepatitis usually resembles type B hepatitis, Purcell and colleagues (NIAID) have reported that epidemic NANB may resemble type A hepatitis. In spite of efforts by many investigators to specifically identify NANB agents, this important objective has not been achieved as yet.

During the past year, members of the U.S.-Japan Hepatitis Panel participated in two conferences. The first, a joint panel meeting with our Japanese colleagues, was held in Hakone, Japan on November 20-21, 1980. The second, an International Hepatitis Symposium, sponsored in part by the NIAID, was held in New York, March 30-April 2, 1981.

Antiviral Substances Program

While vaccines remain the most effective way for controlling viral disease, this approach is not feasible in a variety of viral infections. There is a clear need for chemoprophylactic/therapeutic agents that have a high therapeutic index. The toxicity associated with antiviral drugs remains a major stumbling block to development of effective agents and explains, to some extent, why interferon, as a natural antiviral agent, appears so attractive.

The ASP supports basic research on interferon and antiviral agents through the grant mechanism. As promising antiviral agents are developed, either through research grants or other mechanisms, they must be evaluated in a variety of in vivo systems for efficacy and toxicity. The Program maintains a group of animal model systems which closely mimic human disease in order to rapidly evaluate promising antiviral compounds for toxicity, prophylaxis and therapy. If preclinical studies on a compound indicate potential for use in man, the Program may support clinical trials to determine toxicity and efficacy. A conscious attempt is made to coordinate our efforts with those performed or supported by others (i.e. pharmaceutical firms) and to support those studies that would not ordinarily be done by someone else.

The success of the animal model approach is indicated by the high correlation of positive results in these systems with later results in clinical trials. A major focus during this past year has been studies on genital herpes virus infections in mice and guinea pigs. Although a good model of recurrent infection is not available, studies of latency can be done in these animals. Several compounds have been evaluated for efficacy and toxicity, including acyclovir (ACV), phosphonoformate (PFA), phosphonoacetate (PAA), and bromovinyldeoxyuridine (BVDU). All of these are effective in treatment of HSV-2 infections except BVDU. Efficacious treatment with both phosphonoformate and phosphonoacetate depend on early treatment in both mice and guinea pigs. Antiviral effects begin to wane if treatment is begun more than 24 hours post-infection. While topical application with acyclovir was less effective than either PAA or PFA, it offered the advantage of being able to treat somewhat later in the infection. Bromovinyldeoxyuridine, while showing almost no effect against HSV-2, is very active against HSV-1 and varicella zoster virus. BVDU was tested in a simian varicella virus model and shown to be efficacious at low doses by a variety of routes with no apparent toxicity. This is an exciting compound for future studies because it is effective when given orally even after symptoms appear.

Clinical studies to evaluate antiviral drugs for treatment of severe herpesvirus infections are continuing. A double-blind trial of ara-A, ara-AMP and acyclovir in treatment of herpes encephalitis is in progress. This trial is an outgrowth of an earlier trial which demonstrated that parenteral administration of ara-A reduced mortality of herpes simplex encephalitis from 70 to 28 percent. Further open studies confirmed the original results and indicated that age and level of consciousness were variables influencing outcome. The current trial was initiated to compare ara-AMP (a more soluble form of ara-A) and acyclovir to ara-A in an attempt to further improve recovery from infection. The efficacy of ara-A and acyclovir for treatment of neonatal herpes virus infection is currently being evaluated. A prior double-blind, placebo-controlled trial of ara-A at 15 mg/kg showed substantial effects on morbidity and mortality, but the outcome was still less than desirable. The next stage of the study consisted of an open therapeutic evaluation of ara-A at 30 mg/kg to further document efficacy and evaluate both toxicity and the influence of maternal antibodies on the expression of disease. Acyclovir has the potential for significant further improvement in outcome due to its specificity for HSV infected cells and its high therapeutic index.

A trial of ara-A treatment of herpes zoster infection in immunosuppressed patients has been completed this year and preliminary analysis of the data suggests substantial beneficial effects. Ara-A treatment accelerated healing, decreased progression within the dermatome, decreased the incidence of cutaneous dissemination and visceral complications, and in older patients, decreased pain. Future protocols in this area may attempt to deal with a mode of administration amenable to out-patient therapy and may include normal subjects in the older age group. Additional assessment of the effects of therapy on post-herpetic neuralgia are required.

A substantial proportion of normal individuals suffer from recurrent herpes simplex infections, both oral and genital. It has been estimated that up to 50 percent of the population suffers from herpes labialis, and crude estimates of new cases of genital herpesvirus infections per year range from one quarter to one million. The ASP is currently supporting two double-blind, placebo-controlled trials of patient-initiated therapy of herpes labialis with acyclovir. Initial studies indicated that early treatment was effective in reducing viral titer from day 0 to day 1 but had no positive effects on clinical illness. It is hoped that a higher dose of drug and earlier treatment will improve these results. It is recognized that problems with drug penetration may limit potential benefits of therapy.

Another approach is being pursued in a clinical trial of parenterally administered interferon in females with initial genital herpes virus infections. No results are available since the study is blinded, but it is clear that substantial benefit does accrue to study participants simply due to information and physician involvement gained through participation in the trial.

A double-blind, placebo-controlled trial has been initiated to study treatment of chronic hepatitis B infection. Open studies have indicated that interferon alone does not appear efficacious in males, who represent the major population of carriers. Combined treatment with interferon and ara-AMP, in alternating cycles of 30 days for six months, appears effective in permanent eradication of Dane particles and improvement of liver disease. This regimen will be compared to alternating cycles of ara-AMP and placebo for six months, or placebo alone.

The Program has responded to the anecdotal reports of efficacy of interferon in treatment of juvenile laryngeal papilloma. This devastating disease in its most aggressive form, can require surgery as frequently as every few weeks to maintain an airway. A controlled trial is essential to determine the effects of interferon on tumor regression and recurrences. An RFP was issued in the spring of this year for such a trial.

Support for research on interferon has been a major focus of the Program since its inception. This year the Program has made available high titered sheep antisera to human leukocyte, fibroblast, and mouse interferons. The demand for these reagents is very high; the human and mouse interferon standards are currently being replaced since the supply has been depleted. Requests for immune interferon standard and antiserum to it are frequent. These reagents are now being prepared under contract.

There is increasing public awareness and concern in this country for genital herpesvirus infections. Although no satisfactory epidemiologic data are available, anecdotal reports of an "epidemic" are frequent. The NIAID sponsored a workshop on genital herpesvirus this year in an attempt to review the current focus of efforts in the field, to determine if appropriate approaches are being explored and to develop recommendations for further research. A summary of this workshop will be published in the Journal of Infectious Diseases.

Since acyclovir appears to be a promising antiviral compound for herpesvirus infections, the Institute co-sponsored a meeting with the Burroughs Wellcome Company on this agent. Other meetings of interest were in the area of interferon research. The NIAID serves as a focal point to coordinate efforts and exchange information on interferon among the various institutes. The ASP continues also to be involved in the areas of interferon standards and nomenclature. Several meetings were held this year on these topics.

Much of the basic research on interferon production and its mechanism of action is supported by research grants in the ASP. These studies have demonstrated that the heterogeneity of human interferon is much more extensive than previously thought. The recombinant DNA derived interferons will each have to be compared to native interferons for a variety of antiviral and non-antiviral activities, antigenicities and pharmacological properties.

Bacterial Vaccines Program

Pneumococcal Diseases: Studies sponsored by the NIAID during the past 13 years, along with studies by manufacturers, have led to the development and licensure of the 14-valent pneumococcal polysaccharide vaccine. A collaborative program, coordinated by the Bacterial Vaccines Program, to study the immunogenicity of pneumococcal vaccines in various high risk populations has been under way for the past three years. Approximately 40 studies are included in the program but none receive direct NIAID funding. Antibody assays, however, are performed by the contract-supported reference laboratory at SUNY, Downstate. These studies are nearing completion and have yielded information that will be very valuable to those making recommendations on vaccine usage. Patients with the following conditions appear to mount normal antibody responses to pneumococcal vaccine: juvenile and adult insulin-dependent diabetes, nephrotic syndrome (steriod sensitive), chronic obstructive pulmonary disease, systemic lupus erythematosus (SLE), renal transplant, Hodgkin's disease (before chemo- and radiotherapy), and sickle cell anemia. Patients with SLE but on therapy, hemodialysis patients, renal transplant patients less than six months post-operative, and splenectomy patients seem to mount a more moderate response. Several types of patients, however, respond poorly to pneumococcal vaccine; those with nephrotic syndrome (steriod resistant), chronic renal failure, Hodgkin's disease on immunosuppressive treatment, and multiple myeloma. Further data are needed for patients with bone marrow transplants, leukemia, and other cancers.

Otitis media is one of the most frequent infections of the infant and young child. Fluid may persist in the ears of children for weeks or months following acute infection, and may cause hearing disorders with subsequent learning disabilities. Pneumococci are the bacteria most frequently isolated from the middle ear fluids of children with acute otitis media. A program to study the value of pneumococcal vaccine for the prevention of otitis media was initiated in 1975. Two double-blind efficacy trials of the vaccine, Boston City Hospital and Huntsville (Alabama) Hospital, have recently been completed. The results of the trials have not been completely analyzed. Preliminary analysis indicates that, in the Boston study, vaccine significantly reduced the episodes of otitis media due to types of pneumococci present in the vaccine. Some individual polysacchrides are better immunogens than others and are more likely to be associated with protection. The Huntsville study also showed the vaccine to be associated with a reduction in the number of episodes of otitis due to vaccine types, although not at a significant rate. There was, however, a significant reduction in the number of infants who became otitis prone if they received the vaccine early in life. A Finnish study, not supported by NIAID, has shown a higher level of protection against otitis media by the vaccine than the U.S. studies. A larger Finnish trial is under way and is being done in healthy children rather than otitis-prone children as used in the three other studies.

A third NIAID sponsored project at Vanderbilt University was designed to study dose and booster regimens in infants. The response of infants to the polysaccharides was shown to vary greatly with the individual type. In general, the response of infants to most types is poor.

A chinchilla model was developed under contract support to study pneumococcal otitis media; this work is continuing under a grant. Vaccine studies with this model were previously reported. The model was recently used to study the pathogenesis of pneumococcal disease in chinchillas and the influence of influenza A virus infections on this process. It is clear that influenza infection greatly increases the susceptability of chinchillas to pneumococcal otitis media, pneumonia, and bacteremia.

Meningitis: It is estimated that approximately 20,000 cases of bacterial meningitis occur in the U.S. each year, mostly in young children and infants. For much of the past decade, program efforts have been directed toward development of vaccines for use in children under two years of age where most of this disease occurs. A capsular polysaccharide (polyribose phosphate, PRP) vaccine of *Hemophilus influenzae* was developed through the NIAID program. Although effective in adults and older children, this vaccine has been shown to be non-immunogenic and ineffective in infants. A more recent candidate vaccine containing cell envelope proteins complexed with the polysaccharide has been the disappointing subject of clinical trials and has also been abandoned. We are essentially "back to the drawing board" with *H. influenzae*. Several new contracts and grants are directed at exploring various approaches to the development of a vaccine for use in infants. Approaches include: (1) extraction and characterization of outer membrane proteins as potential antigens, (2) preparation of complexes of pure PRP with various pure proteins such as diptheria toxoid, (3) preparation of molecules of PRP containing increased numbers of carboxyl groups to increase immunogenicity, (4) use of cross-reacting polysaccharides such as those from *E. coli* K-100, and (5) isolation of temperature-sensitive mutants of *H. influenzae* to be used as live, whole-cell vaccines.

A *H. influenzae* vaccine in which the *H. influenzae* PRP is complexed with whole pertussis cells has been developed by Lederle Laboratories. Phase I and II studies by Lederle indicate that this preparation stimulates *H. influenzae* PRP antibodies in infants. The NIAID may assist with support of clinical trials of this vaccine and has begun support of Lederle studies to identify the subcellular fractions of pertussis which provide the adjuvant effect.

In addition to efforts to improve the infant's response to bacterial meningitis vaccines, small scale studies continue on the protection of young infants by hyperimmunization of the mother. *H. influenzae* PRP vaccine has been given to women prior to pregnancy and in cases in which the mother responds well to the vaccine, comparable levels are seen in the cord bloods and in the infants. The children are being followed to see how long the antibody lasts and whether or not it interferes with the ability to respond to the vaccine later in life.

Groups A and C meningococcal polysaccharide vaccines have been licensed as a result of efficacy studies in adults and older children. The group A vaccine was shown to be effective in children down to three months of age in an efficacy trial in Finland sponsored by the NIAID. Group C meningococcal polysaccharide vaccine is poorly immunogenic in very young children. A capsular polysaccharide extracted from a variant group C strain has been prepared at Rockefeller University. This new material is of a different chemical structure than the licensed group C vaccine, but it is immunologically cross-reactive. Through the NIAID program, this vaccine has been shown to be superior in adults and older children. Studies in infants are under way.

Group B meningococcal polysaccharide is non-antigenic in humans. Two candidate vaccines composed of purified proteins from the outer membrane layer of the organism were developed at the BoB/FDA and the Naval Medical Research Institute. These have been studied in humans during the past three years under our program; the results have been disappointing and work with these preparations has ceased. A new candidate vaccine preparation developed at BoB which is a complex of the outer membrane proteins and a high molecular weight group B polysaccharide is being tested in our program at the present time. Results in adults and adolescents are very exciting and clinical studies in younger children and infants are planned for this fall.

Rocky Mountain Spotted Fever: The presently licensed vaccine for RMSF is not considered effective and is no longer commercially available. The NIAID has assisted in the past with clinical studies of a new whole cell inactivated vaccine developed by the Army (USAMRIID). An efficacy trial of this vaccine using challenged volunteers has recently been completed at the University of Maryland (see report from Clinical and Epidemiologic Studies Branch). Studies on the epidemiology of RMSF in North Carolina and Maryland have been organized, and data are being collected on incidence of disease and the role of domestic animals as vectors. In the event a promising candidate vaccine is developed, it may be possible to do field trials in these populations. Research at the Rocky Mountain Laboratory on new subcellular vaccines looks promising. We will assist with clinical trials of candidate vaccines if results warrant testing in humans.

Pertussis: Pertussis is an area of considerable interest to program staff because the current vaccine, while effective, is sometimes responsible for rather serious reactions. Considering the advances that have been made in the past 10-15 years in the development of subcellular bacterial vaccines, it would seem that the technology is such that an improved pertussis vaccine can be developed. Although there are no program efforts in directed research for development of new pertussis vaccines, several grants are looking at the role of various bacterial components in pathogenesis and at the nutritional requirements for maintenance of antigenically stable pertussis strains. Very interesting research is under way at the BoB, Rocky Mountain Laboratories, and the Japanese NIH on subcellular components with vaccine potential. We will monitor this work and assist with clinical studies when appropriate.

Viral Vaccine Program

The Viral Vaccine Program covers those areas where research should be stimulated toward the goal of an effective vaccine, but are not covered by the other specific programs. As reported last year, New York University is evaluating an attenuated varicella vaccine, under contract, in immunosuppressed children. Humoral and cellular immune responses in fifteen normal adults have been analyzed. Three of the adults have had close exposures without contracting varicella.

Twenty leukemic children have received the V-Z vaccine. Thirteen were off chemotherapy for two to six months. Six had chemotherapy stopped for two weeks. The only possible side effect has been that two children with chemotherapy suspended developed maculopapular lesions about one to two weeks after vaccine. V-Z virus was not isolated from them, nor has shedding virus been documented in any vaccinee. All vaccinees tested have responded immunologically.

The Development and Applications Branch also supports five General Vaccine Evaluation Units that form the backbone of the Branch's efforts for approaches to the control of important disease problems. Each unit has specialized population groups available to them, i.e., normal adults, pediatric groups, "at risk" adults and/or children, for evaluating viral vaccines, bacterial vaccines, antiviral drugs, and environmental controls. Epidemiologic studies performed by several of the units have provided much of our current knowledge on the natural history of several of the respiratory agents.

MOLECULAR MICROBIOLOGY AND PARASITOLOGY BRANCH

The Molecular Microbiology and Parasitology Branch plans and conducts research grant, program project grant, contract, training grant, fellowship and career award programs in molecular microbiology, (biochemistry, genetics, DNA recombinants, physiology), and parasitology and medical entomology. It also coordinates the activities of the Parasitic Diseases Panel of the U.S.-Japan Cooperative Medical Science Program.

Approximate Level of Support

Molecular Microbiology Section

Activity	Number	Amount
Research Grants [1/]	229	$17,405,596
Research Contract	1	337,903
Training Grants	4	371,680
Fellowships	41	566,543
Subtotal	275	$18,681,722

1/ Includes 14 Career Awards ($506,185)

Parasitology Section

Activity	Number	Amount
Research Grants [2/]	218	$14,561,336
Program Project Grants	2	890,053
Research Contracts	3	199,448
Training Grants	9	668,812
Fellowships	13	261,663
Sub-total	245	$16,581,312

2/ Includes 6 Career Awards ($195,929)

Branch

Activity	Number	Amount
Research Grants [3/]	447	$31,966,932
Program Project Grants	2	890,053
Research Contracts	4	537,351
Training Grants	13	1,040,492
Fellowships	54	828,206
TOTAL	520	$35,263,034

3/ Includes 20 Career Awards ($702,114)

Subsequent to submission of the Annual Report for FY 1980 several meetings occurred which were not covered in the report.

The first of these, held between July 29 and August 1, 1980, in Tsukuba City, Japan was the 15th Joint Conference on Parasitic Diseases of the U.S.-Japan Parasitic Diseases Panels. Dr. Delappe took the place of Dr. Phifer at the meeting. A full report was made following the meeting.

Dr. Phifer attended the August, 1980 meeting of the American Society of Parasitology in Berkeley, California.

Dr. Delappe attended the Sept. 21-25, 1980, meeting on Structure & Expression of Eukaryotic Genomes held by the French Institut National de la Santé et de la Recherche Medicalé (INSERM) at Blois, France.

Early in FY 1980, Dr. Hamilton Smith, Nobel Laureate, of The Johns Hopkins University was asked by Dr. Delappe to address the annual meeting of the Mid-Atlantic Extrachromosomal Elements Group held on Sept. 26-28, 1980 at the Boar's Head Inn near Charlottesville, Virginia. Dr. Delappe introduced him.

Early in FY 1981 (Oct. 20-22, 1980) the Branch was represented at a combined Rockefeller/Macy Conference in New Orleans, La. on the Present Status and Future of Parasitology. Dr. Delappe presented a paper on "Research in Parasitology from an NIH Perspective" that will be published shortly in Proceedings of the Josiah Macy, Jr. Foundation.

In October, 1980, the proceedings of our Workshop on Application of Recombinant DNA Technology was published as a supplement to the September, 1980, issue of the American Journal of Tropical Medicine and Hygiene.

In December 1980 a book was published by the Czech Medical Press containing the proceedings of the 4th International Symposium on Antibiotic Resistance, a conference held almost a year and a half before (June 3-8, 1979) in Bratislava, Czechoslavakia.

A poster presentation was given at an International Conference on Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids held in Santo Domingo, Dominican Republic - January 5-9, 1981. Plenum Press will publish the proceedings.

A workshop on Medically Important Parasites was held at Cold Spring Harbor Laboratory, N.Y. on April 7-8, 1981. Dr. Delappe spoke on the importance of transfusing parasitology, medical entomology and tropical medicine with molecular biology, including immunogenetics and immunochemistry.

On June 16, 1981 a parasitology conference was held at the Rockefeller Foundation in New York City. This was a "follow-up" on the October, 1981 New Orleans meeting sponsored by the Macy & Rockefeller Foundations. The purpose of the conference was to gather together a representative group of foundation people to interest them in supporting the "great neglected diseases" of mankind and domestic animals. Dr. Delappe spoke on "The Present State of Parasitology."

During the period June 28-July 3, 1981 Dr. Delappe attended a Gordon Conference on Bacterial Cell Membranes in New Hampton, N.H. From August 3-7, 1981 another Gordon Conference will be attended, this one on Immunological and Molecular Aspects of Parasitism, at New London, N.H.

Dr. Kenneth Phifer, Parasitology Program Officer of this Branch, retired on May 29, 1981. Dr. Harley Sheffield has been chosen to replace him. On August 10-14, 1981, Dr. Sheffield will attend the American Society of Parasitology meeting in Montreal, Canada.

During the period September 22-27, 1981, Dr. Delappe will attend a meeting funded by the Rockefeller Foundation of the 14 units of the "great neglected diseases program" at Magdalen College, Oxford, England.

MOLECULAR MICROBIOLOGY

Program Summary

In addition to the basic free-ranging research of relevance to the Institute, there are two structured programs supported by this section of the Branch, one of which involves mechanisms of resistance to antimicrobial agents and the other, recombinant DNA.

(1) Mechanisms of Resistance to Antimicrobial Agents

During the past eleven years, the problem of microbial resistance to therapeutic agents has become increasingly apparent. The relevance of this phenomenon to other interests of the Institute such as hospital-associated infections, gram negative bacteria, and other organisms, together with investigations of gonococci and pneumococci, is obvious.

There is substantial clinical and epidemiological evidence that development of antibiotic resistance, and especially plasmid (extrachromosomal) mediated drug resistance, represents a growing problem in medical care. Most of the projects are concerned with defining the fundamental biological mechanisms involved in the development of drug resistance by microorganisms. Specific goals involve investigation of the origin, development, evolution, expression and mechanism of drug resistance in a variety of specific microorganisms. Examples of microorganisms of particular interest are (but not limited to): *Haemophilus influenzae*, *Enterobacteriaceae*, *Pseudomonas*, *Neisseria*, staphylococci, streptococci, mycobacteria, mycoplasmas, and pathogenic fungi.

Research Highlights

AI 10311-12 D. Dubnau (Public Health Research Institute of New York): Resistance to the macrolide-lincosamide-streptogramin B (MLS) group of antibiotics is widespread and of clinical importance. This researcher reports that 10 of 15 natural isolates of *Bacillus licheniformis*, a common soil organism, are resistant to the MLS antibiotics. The resistance determinant from one of these strains was cloned onto a *B. subtilis* plasmid vector, and the resulting hybrid plasmid (pBD90) was used to prepare radioactive probe deoxyribonucleic acid for hybridization studies. All of the resistant *B. licheniformis* strains studied exhibited homology with the pBD90 insert. Plasmid pBD90 showed no homology to the following staphylococcal and streptococcal MLS-resistance plasmids: pE194, pE5, pAM77, pI258. Plasmids pE194 and pE5, on the other hand, carry homologous MLS genes but showed no detectable homology to one another in their replication genes. pBD90 specified a 35,000-dalton erythromycin-inducible protein, detectable in minicells, which therefore appears different from the 29,000-dalton inducible resistance protein specified by pE194. He concludes that there are at least three distinct MLS resistance determinants to be found among gram-positive bacteria.

AI 15619-03 V. Burdett (Duke University): Since 1957, there has been a significant increase in the incidence of tetracyline resistance among clinical isolates of *Streptococcus* and tetracycline resistance is now commonly encountered. Dr. Burdett examined 30 tetracycline-resistant clinical isolates of group B *Streptococcus* to assess the extent to which tetracycline resistance is plasmid-mediated. Of these, 27 showed no physical or genetic evidence of plasmid-mediated resistance; however, one conjugative and two small (3.5 x10^6-dalton) multicopy non-self-transmissible tetracycline resistance plasmids were identified. The conjugative plasmid was transmissible to *Streptoccus faecalis* as well as to *Streptococcus agalactiae* (group B). The two nonconjugative plasmids were readily mobilized by a number of sex factors into these same two backgrounds and, in addition, readily transformed *Streptococcus sanguis* Challis to tetracycline resistance. Due to readily available sites for several site-specific endonucleases, these small, multicopy plasmids should prove useful as cloning vehicles in this host system.

AI 12500 J.R. Murphy (Harvard Medical School): This worker continues his studies on virulence factors of *Corynebacterium diphtheriae*. During the past year he has been successful in the isolation and preliminary characterization of a 43 clone library of monoclonal antitoxin-producing cell lines. Balb c mice were immunized with diphtheria toxoid. Following a rise in anti-toxin the mice were sacrificed and their spleens removed and perfused with tissue culture medium. Spleen cells were then fused with NS-1 cells in the presence of polyethylene glycol, and hybridoma cell lines selected in HAT media. Interestingly, 9 clones that produce antibody produce an antibody that is not neutralizing. These hybridoma lines will be important in the study of diphtheria toxin entry into eukaryotic cells.

AI 07877-14 R.J. Collier (University of California, Los Angeles): Continuing his studies on molecular mechanisms of bacterial pathogenesis, Dr. Collier and co-workers have constructed and characterized a hybrid toxin containing the A chain of diphtheria toxin linked via a disulfide bridge to the B protomer of cholera toxin. Cholera toxin B protomer, previously derivatized with 4-5 cystaminyl groups per pentameric protomer, was reacted with reduced diphtheria toxin chain A to give the desired hybrid, containing an average of 2 molecules of diphtheria toxin chain A per cholera toxin B protomer. A concentration of 0.3 nM hybrid inhibited protein synthesis by 50% in 24 hours in several cultured cell lines; thus the hybrid was about 10-fold more toxic than of a (diphtheria toxin chain A)-SS-(concanavalin A) conjugate described previously. Evidence was obtained that toxicity of the hybrid was dependent on the functional contributions of both the diphtheria toxin chain A and cholera toxin B protomer moieties.

AI 14149-02 H.E. Gilleland (Louisiana State University Medical Center): The principal investigator has continued his studies on the outer membrane of *Pseudomonas aeruginosa*. One of the three basic mechanisms by which bacterial cells become resistant to antibiotics is alteration of the permeability properties of the outer membrane of the gram-negative bacterial cell envelope so that the antibiotic is excluded from entry into the cell. To date this mechanism has remained poorly understood and not comprehensively studied. This research endeavors to explain how an important human opportunistic pathogen, *Pseudomonas aeruginosa*, modifies its outer membrane to acquire resistance to the antibiotic polymyxin B. The results indicate that lipid alterations in the cell envelope play an important role in mediating polymyxin resistance and suggest that loss of receptor phospholipids which bind polymyxin may be the key alteration behind polymyxin resistance. Further studies will seek to clarify the role played by the various outer membrane components and should lead to a greater understanding of the role of the cell membrane in antibiotic resistance.

AI 16370-01 P.S. Cohen (University of Rhode Island): Dr. Cohen is embarking on a study of the molecular basis of *Escherichia coli* colonization. He has isolated several mutants of human fecal and K12 *E. coli* strains which have much reduced colonization abilities relative to their parental strains. Furthermore, he has isolated two human fecal strains of *E. coli* each of which has lost a colicin plasmid and each of which is a far poorer colonizer than its immediate parent. These strains all grow in a minimal medium and all colonize the mouse intestine at high levels when fed to mice individually. It is therefore doubtful that the reason for their reduced colonization abilities relative to their parents is nutritional. These organisms and their parents should therefore serve as the means by which the molecular basis of large intestine colonization is determined, according to Dr. Cohen.

(2) Recombinant DNA Molecular Research

During the past few years, the development of certain techniques in the area of molecular biology has made it possible to construct functional DNA molecules *in vitro* which contain segments derived from diverse biological sources. The scientific innovations which led to this technological breakthrough were mostly derivative from basic studies on the mechanism of restriction, which normally acts as a barrier to gene flow among microorganisms, and on the molecular biology and genetics of bacterial plasmids, especially those specifying drug resistance. Grantees of this Institute played a preponderant role in both of these areas. Whereas DNA recombination in nature has depended on random processes, the experimental techniques now available enable the *in vitro* construction and subsequent replication of DNA molecules needed for specific experimental goals. This basic advance, coupled with refinements of the existing technology, should provide greater knowledge of the mechanisms of pathogenicity (at the molecular level) of viral, bacterial, mycotic and parasitic agents. This information, in turn, may lead to improved prevention, diagnosis and treatment of infectious diseases. Notwithstanding the potential benefits of recombinant DNA molecule research, there may be associated potential biohazards which must be avoided by the design, construction and testing of safer host-vector systems for use in these studies.

The primary goal of this research is the development and utilization of recombinant DNA molecule technology to increase fundamental knowledge and ultimately enhance control of the etiological agents of infectious diseases. Another goal is the synthesis of a variety of biologically useful substances through the construction of bacterial cells containing functional DNA of either plant or animal origin. An equally important goal is the identification, assessment, and elimination of any and all potential biohazards encountered in the exploitation of this technology.

Research Highlights

AI 10106-11 J. Konisky (University of Illinois): Dr. Jordan Konisky has further investigated colicins, substances produced by intestinal bacteria with which they wage war for living space with other bacteria. Colicins are really antibiotic in nature, and from time to time the question has arisen concerning the immunity of a given bacterium to the deleterious effect of its own colicin. A possible explanation of this phenomenon is that a particular plasmid which makes a colicin may also have a gene that encodes for production of a protein that gives it immunity to its own colicin. The protein probably exists at the bacterial surface membrane, and the immunity is exercised at the same location. Dr. Konisky has determined that two separate genes code for colicin production and immunity. He split off these two genes from one another utilizing recombinant DNA technology and got two separate plasmids - one that possessed the gene coding for colicin and another that coded for immunity to the colicin. When he reintroduced the colicin producing plasmid without the immunity-producing plasmid into the bacterium, the bacterium was "knocked off" by its own colicin!

AI 11479-09 H.G. Khorana (Massachusetts Institute of Technology): He has studied a Halobacterium which contains a light-sensitive pigment, bacteriorhodopsin. He has identified and studied the specific gene which produces this substance. He has also sequenced the nucleotides in the gene and the amino acids produced by the nucleotides. He has removed the lipid content from a protein ("retinal") produced by these amino acids, has frozen and stored it and determined that it has seven helices. He has denatured the protein and then renatured it from the fragments obtained, and studied its spatial orientation.

AI 8619-14 S.N. Cohen (Stanford Medical School): Dr. Cohen has perfected a DNA cloning system for interspecies gene transfer in antibiotic-producing Streptomyces. The ability to transfer genes among different Streptomyces species and to obtain phenotypic expression in the new host is of potential importance in the use of DNA cloning methods for the development of new antibiotics by the introduction of exogenously derived enzymes that modify pre-existing antibiotics or lead to the synthesis of hybrid antibiotic molecules. In addition, the cloning on multicopy plasmids, such as pSLP101, of genes involved in rate-limiting biosynthetic steps may prove useful in increasing the yield of antibiotics produced by Streptomyces species.

(3) Other Basic Research

AI 04156-20 J.B. Neilands (University of California - Berkeley): Dr. Neilands continues his work on iron containing compounds (siderophores) and their metabolism in microorganisms. Clinical isolates of invasive strains of Escherichia coli frequently carry the colicin V plasmid. Recently it has been found that the virulence factor is not attributable to the production of colicin V per se, but, rather, to the synthesis of an hydroxamate type siderophore by the plasmid. This siderophore is known as aerobactin. The main significance of this work is that it identifies a specific siderophore, aerobactin, as a virulence factor in enteric bacteria pathogenic in humans. While virulence is undoubtedly dependent on a multitude of determinants, it is clear that the capacity of the invading cell to harvest iron from the host is one of the important attributes of the invasive microbe. This basic research provides the opportunity to launch a program of rational drug development in which aerobactin analogues are synthesized and tested for acceptability to the aerobactin transport system.

AI 01278-25 K.L. Rinehart (University of Illinois): Dr. Rinehart and co-workers have isolated a new class of depsipeptides from a tunicate (ascidian, sea squirt) collected on the ocean floor in the Caribbean Sea. These are didemnins, two of which, Didemnin A and Didemnin B show special promise. Didemnin A has antiviral activity inhibiting both RNA and DNA viruses, while Didemnin B may be more important in inhibiting the growth of leukemic cells. The latter is a derivative of Didemnin A and it also inhibits viral replication in vitro and P388 leukemia in vivo. Currently the didemnins are being tested at Upjohn Company where it has been established that they provide a rather effective treatment for ordinarily lethal doses of vaginal Herpes simplex, Type II in mice. Presently they are trying to establish an optimal dose regimen for the drug. Before it can be tested in humans, however, a good bit of toxicology needs to be carried out with respect to carcinogenicity and teratogenicity as the compounds are highly cytotoxic.

PARASITOLOGY

Program Summary

In addition to the basic free-ranging research of relevance to the Institute, there are two structured programs supported by this section of the Branch - biological regulation of vectors and immunology of parasitic infections.

(1) Biological Regulation of Vectors

This program has as its goal the advancement of fundamental studies which might lead to effective methods of biological regulation of vectors. For the past 30 years, control of pests and disease vectors has been based primarily on the use of synthetic organic compounds which had the "advantages" of long residual action and toxicity to a broad spectrum of target organisms. It has now been shown that, because of these very characteristics, many of these pesticides are more deleterious than beneficial when all effects on man and his environment are considered. Furthermore, resistance to broad spectrum chemical pesticides has reduced their effectiveness in many vector control programs. For these reasons the search for alternative methods of pest control has become imperative, and it is generally agreed that the best approaches will consist of integrated pest management programs which combine biological control, in the broadest sense, with the judicious use of more specific chemicals and management of the physical environment. This approach to vector control must be based on adequate information about the ecology of the target organisms in which the control program is to be conducted, effects of control measures on non-target organisms in the environment, and the biology of the disease organism being transmitted.

Research Highlights

AI 14520-03 M. Trpis (Johns Hopkins University). Experiments on cytoplasmic incompatibility suggest that the factor responsible for this phenomenon is the presence of Rickettsia type microorganisms in several Aedes species. Treatment of mosquitoes susceptible to infection with the Brugia filaria parasite with heat, tetracycline and chloramphenicol resulted in the change of 100% susceptible mosquitoes into refractory ones. Subsequent generations survived as refractory vectors. If this refractoriness becomes a permanent trait, this characteristic might be a valuable tool in the control of filariasis.

AI 17024-02 A.J. Domnas (University of North Carolina). Field studies have shown that the fungus Lagenidium giganteum has potential as a biological control agent for mosquitoes which are important vectors of human disease. At low levels this fungus can operate effectively against Culex quinquefasciatus which serves as a vector of human filariasis and viral encephalitis.

AI 15605-03 D. Molloy (University of the State of New York). Laboratory assays have indicated that infective-stage juveniles of the nematode Neoaplectana carpocapsae are highly pathogenic to Simulium larvae. These results show that these nematodes have potential value as agents in the biocontrol of black flies, the vectors of onchocerciasis.

AI 12772-03 B. Federici (University of California, Riverside). Field tests run in the San Joaquin Valley of California have proved Bacillus thuringiensis israelensis (BTI) effective in controlling Aedes and Culex mosquitoes with applications as light as 1/4 to 1/2 pound per acre. Even in hard-to-treat areas such as rice paddies, BTI was effective. In addition to killing mosquito larvae, BTI also attacks the larvae of the black fly, the vector of onchocerciasis. An experimental use permit has been issued and full registration by EPA is expected.

(2) Immunology of Parasitic Infections

The complexity of structure and function of parasites has made the study of the immunology of these infectious agents exceptionally challenging and rewarding. Exciting opportunities for the elucidation of mechanisms and manifestations of immunological responses to parasites now exist as the result of the impressive developments in immunology in recent years. Major ultimate goals of studies on the immunology of parasitic infections are the development of effective vaccines for the prevention of parasitic diseases (such as malaria, schistosomiasis, and filariasis), the intervention in the host response to prevent or ameliorate disease processes which are immunologically mediated, and the development or improvement of immunodiagnostic procedures for parasitic infections, especially as they relate to the immune status of the host.

A related goal of these studies is to contribute to an understanding and solution of basic and clinical problems associated with other disease entities, especially immunological disorders and hypersensitivity states. A number of parasitic infections are excellent models for such studies as (a) the mechanisms of intracellular immunity, (b) the enhancement of suppression of concurrent infections or tumor development, (c) immunopathological mechanisms, (d) development of disease process in immunosuppressed or immunostimulated hosts, (e) the biochemical and genetic mechanisms for the development of pathogen variants with different immunolgical characteristics, (f) the genetic basis for variations in host response, and (g) the role of IgE and other cytotropic antibodies in hypersensitivity.

Research Highlights

AI 16639-01 A. Vincent (University of South Florida). This young investigator in the first year of his grant has shown that the congentially athymic or nude mouse is quite susceptible to infection with the filarial nematode *Brugia pahangi*, whereas the normal mouse is quite resistant to infection with this parasite. These studies suggest that resistance to *B. pahangi* is thymus-dependent and can be restored to athymic mice through reconstitution. He is currently studying the role of various immune components in the protective responses against filarial larvae.

AI 04717-19 J. Remington (Palo Alto Medical Research Foundation). Treatment of intact *Toxoplasma* tachyzoites with individual mouse monoclonal antibodies to *Toxoplasma* which are directed against single membrane antigenic determinants facilitated the phagocytosis of the parasite and prepared it for intracellular destruction by non-elicited mouse peritoneal macrophage. In those instances in which the organism survived intracellularly, their multiplication was significally reduced. Such monoclonal antibodies should be useful in further elucidating the role of antibody in resistance to *Toxoplasma* and other facultative and obligate intracellular organisms.

AI 16479-02 J. David (Harvard University). Monclonal antibodies have been produced which recognize species-specific determinants of *Leishmania mexicana* and *L. braziliensis*. These should be especially useful in allowing rapid diagnosis of these species in the field, since those infected with *L. braziliensis* could be selected for intensive therapy in a hospital. Further, the monoclonals produced by *L. braziliensis* appear to recognize a number of different antigens, and the organisms of *L. braziliensis* appear to occur as a number of subspecies.

AI 13366-05 R. Nussenzweig (New York University). Monoclonal antibodies directed against a surface component of *Plasmodium berghei* sporozoites confer complete protection to mice against a lethal inoculum of parasites. The degree of protection is a function of the number of parasites used in the challenge and of the antibody concentration in serum. These studies suggest that antibodies interfere with a parasite function necessary for its infectivity such as the ability to penetrate into the target cell or to multiply in the hepatocytes.

AI 14185-03 A. Sher (Harvard University). In a study of the function of effector cells which have been implicated in the mechanism of schistosmulum killing, it was found that activated macrophages are probably more potent effector cells than eosinophils in damaging the parasite *in vitro*. It was also shown that cytotoxic T lymphocytes can recognize schistosomula but are unable to kill them. This investigator recently joined the Laboratory of Parasitic Diseases, NIAID.

AI 1651501 R. Crandall (University of Florida). For comparison of antibody responses during *Brugia* infection of Rhesus *Patas* monkeys the enzyme-linked immunosorbent assay (ELISA) has proven an economic, convenient and sensitive test and the results suggest that antibody responses to antigens of the developmental stages of the parasite do vary with the course and "clinical" type of infection. The use of isolated, characterized antigens from the parasite stages promises to provide a more critical comparison of antibody responses.

(3) General Parasitology Research Highlights

AI 17340-01 C. Bacchi (Pace University). An inhibitor, alpha-difluoromethylornithine, which was developed as a chemotherapeutic agent for diseases characterized by rapid cell proliferation, has been shown to block the formation of polyamines in trypanosomes and to cure experimental laboratory infections. The pharmacology of this compound is such that it can be given orally, shows no toxic side effects and does not seem to provoke resistance.

AI 09625-12 P. Weinstein (University of Notre Dame). In studies of the *in vitro* development of the filarial parasite *Dipetalonema viteae*, third stage larvae were taken from ticks and placed directly into *in vitro* cultures. These have developed and undergone molt to the fourth state. Development of these techniques of *in vitro* culture under axenic conditions should provide important tools for the production of antigens for immunization and diagnostic purposes, for the screening and testing of potential chemotherapeutic agents and for physiological and metabolic studies.

AI 16644-02 D. Lindmark (Cornell University). Many gaps in our understanding of the basic biology of *Giardia* have been filled during the past grant year. It has been shown that the metabolism of *Giardia lamblia* is similar to that of trichomonads and many species of *Entamoeba*. Carbohydrates and endogenous reserves are incompletely oxidized to organic end products and CO2 by a flavin, iron-sulfur protein-mediated fermentative pathway with energy produced by substrate level phosphorylation. The mechanism of metronidazole against *G. lamblia* involves a pyruvateferredoxin oxidoreductase which reduces a low oxidation-reduction potential electron acceptor which, in turn, chemically reduces metronidazole, forming a toxic product.

AI 15663-03 J. Marr (St. Louis University). Thiopurinol and its ribonucleoside were found to be effective *in vitro* against both the intracellular and extracellular forms of *Leishmania donovani* and the extracellular forms of *L. braziliensis* and *L. mexicana*. These findings suggest that the mechanism of action of the pyrazolopyrimidines in the pathogenic hemoflagellates resides not in their ability to be incorporated into RNA but probably because they, or their ribonucleotide products, inhibit one or more enzymatic steps in purine metabolism.

(4) Contract Activity

The schistosomiasis supply contract at the University of Lowell has continued to be utilized extensively by most schistosomiasis researchers in this country. The contract provides all three human schistosome species and their vector snails.

The filariasis supply contract at the University of Georgia provides research workers with five genera of filariae as well as the appropriate vectors. Acceleration of research on filariasis has been largely attributable to this service.

A contract at Cornell for the development of techniques for the radiolabelling of schistosome and filarial larvae involves the interaction and collaboration of experts in a variety of disciplines - parasitology, entomology, radiobiology, immunochemistry, and immunoparasitology. Slow but definite progress is being made in the attempt to solve a very difficult yet significant problem in parasitic diseases.

EXTRAMURAL ACTIVITIES PROGRAM

NIAID

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REPORT OF THE DIRECTOR, EAP

A. Introduction

Fiscal Year 1981 was a year of significant changes in the Extramural Activities Program brought on to a large extent by modification of responsibilities of key staff. The departure of Dr. William Gay, Director, EAP since 1971, in November; Dr. Robert Byrne's stepping in as Acting Director, EAP, but subsequently being stricken by a heart attack; the designation of Dr. Froehlich as Acting Director, EAP, and Dr. Jack Whitescarver as Acting Deputy Director, EAP, in addition to their other duties -- have thankfully not created any obvious disruption of EAP activities.

Grants and Contracts were awarded in a timely manner, with the Contracts Management Branch strictly adhering to NIH-set competitive procurement goals. Grants Management staff adjusted admirably to the vagaries and uncertainties of the 1981 budget as did the Fiscal Management Section. The effects of a severe cut in the contract budget has been courageously addressed by the Contracts Management staff.

The Program and Project Review Branch continued to have a busy review schedule, and all deadlines were met in terms of timely preparation of site visit reports, summary statements and the usual detailed minutes of committee meetings. There is cooperative endeavor among program, contracts management, and review staff in achieving the objective of having as many as possible of the competitive proposals reviewed by the chartered committees. The processing of conference and workshop proposals, formerly coordinated by the Fogarty Center, was shifted to the Division of Research Grants, thus converting all such proposals to grant applications (R13s). This Institute subsequently adopted a policy of having these R13 applications reviewed by the chartered committees. As with all BIDs, R13s are secondarily reviewed by Councils/Boards following initial review.

Procedures for Council preparation were streamlined to make maximal use of a chronically understaffed Review Services Unit (RSU) and to cut down on paperwork. Support staff from other branches of the EAP were temporarily assigned to the RSU during peaks of activity in preparation for Council meetings. The number of Council Books (books of all summary statements) prepared for staff was drastically reduced as were those available for Council members, with no adverse effects. More concentrated effort and attention was paid to the preparation of Special Problem Workbooks, around which most of the Council grant-related discussions revolved and an ample supply was prepared for concerned staff.

Staff of the Data Control Section ably carried on their duties, often under trying conditions. The scientific classification (coding) of grant applications, heretofore made on all applications received, was limited to only fundable applications, significantly reducing the workload of Program Analysis staff, allowing them to accomplish other related work in timely manner.

The NIH, beginning this year, set a goal of funding 5,000 competing research grants and supporting 10,000 trainees. It also begun paying by raw, rather than by normalized scores. In the regular research grant category, the NIAID funded competing applications with scores of 178 or better, as well as 31 selected applications beyond the cutoff which were of high programmatic relevance. Only twelve competing training grant applications were paid this year, as were 42 competing individual fellowship applications. We are aiming to pay up to 423 research competing projects this year, for a pay rate of 33%.

B. Personnel

There were eleven departures form the Extramural Activities Program in FY 1981, among them key staff. Dr. William Gay, Director EAP since 1971 moved in November, 1980, to the Division of Research Resources to direct their Animal Resources Program. Dr. Robert Byrne then served as Acting Director EAP until he was stricken in mid-January with a heart attack. (After a prolonged illness Dr. Byrne died on July 8, 1981.) Since January 22, Dr. Froehlich has been serving as Acting Director EAP, concurrently continuing her responsibilities as Chief, Program and Project Review Branch. Dr. Jack Whitescarver, in addition to his duties as Special Assistant to the Director, NIAID, was designated Acting Deputy Director, EAP. In this capacity he was given charge of the Program Analysis and Evaluation Branch. The Review Services Unit continues to be understaffed and the position of Head, Review Services Unit, has remained vacant since October 1980. There has been a turnover of executive secretaries in the Program and Project Review Branch. Dr. Thelma Fisher, executive secretary of the MIDAC, moved to the NIADDK to manage the review of that Institute's Diabetes Centers. Dr. Harley Sheffield, executive secretary of the AIRC and TBIC will be moving over to head the Parasitology Program, in Dr. Irving Delappe's Branch. Ms. Darlene Michalski, Committee Management Assistant (CMA), NIAID, has moved to NICHD for a comparable job but at a higher grade level. Continuing efforts on the part of the Acting Director, EAP to convince Personnel Office of the crippling effects the low grades have had on the activities of the Committee Management Office and the Review Services Unit has finally resulted in the two positions, CMA and Head, Review Services Unit, being upgraded to grades comparable to those of other BIDs. Ms. Margery Grubb, whom the NIAID temporarily lost to NIGMS, will become the NIAID Committee Management Assistant beginning August 9, 1981. Ms. Lillian Myers, secretary to the Director EAP since 1971, retired on January 18, 1981 and has not been replaced. Dr. Louis Bourgeois, NIAID training officer since 1972 is retiring in early August and will be replaced by Dr. William Bennett, formerly of the Health Resources Administration. Following this change, the Office of Research Manpower will be transferred to the OD-NIAID.

Two new positions were added to the Grants Management Branch, that of secretary and grants clerk. Dr. Susan Spring replaced Dr. Fisher as executive secretary, MIDAC. There is active recruitment for Dr. Sheffield's replacement. Ms. Lai Tan has replaced Ms. Jo Ann Holmes in the Program

Analysis Section. Ms. Jo Ann Stesney, formerly Head Program Analysis Section, is now Assistant to the Acting Director, EAP, performing a variety of duties requiring scientific knowledge and a familiarity with NIAID programs. Ms. Mildred Warfield, although no longer in charge of the review of conference proposals (executive secretaries now prepare the summary statements on R13s) has been given the new responsibility of coordinating Council activities and Institute liaison for R13s.

Full-time Permanent Employees who have left EAP

Louis D. Bourgeois
Robert J. Byrne
Thelma N. Fisher
William I. Gay
Constance Guernieri
Jo Ann Holmes
Etta Kidwell
Darlene Michalski
Peter A. Mullin
Lillian W. Myers
Harley G. Sheffield

New Full-Time Permanent Appointments

Linda M. Blazer
Lila Cave
Joyce F. Lopez
Susan B. Spring
William J. Stalters
Lai S. Tan
Margery Grubb

Awards

Anne S. Rabon
Marietta C. Robinson
Hubert L. Sumner III
Gary E. Thompson
Fredric H. Wiener

CMB Group Award

FY 1980 (Not Mentioned in Annual Report

Lou Pollack - Directors's Award
Rosalie Strauss
Grace Ellis
Louis Bourgeois

C. Manpower Development

During FY 1981 two hundred and thirty-one (231) individual fellowship (F32) applications were reviewed, of which 197 or 85 percent were approved (Table I). These figures do not show a significant difference from those of last year.

Table I
NIAID Individual Fellowship (F32) Applications Reviewed FY 1981

Total No. Applications	Applicants by Degree		Approved Applicants	
	M.D.	Ph.D.	M.D.	Ph.D.
231	44(4)	187(8)	35(3)	162(7)
	(19%)	(81%)	(15%)	(70%)

Numbers in ( ) = number of senior fellows (F33s)

Fifteen percent of approved applicants were M.D.s and 70 percent were Ph.D.s

It is estimated that about 40 of the approved applicants will be awarded by the close of the fiscal year.

Table II shows the number by program, of trainees requested and the number recommended for support in the institutional applications (T32) reviewed.

Table II
Total No. Trainees Requested and Recommended for 1st Year FY 1981

Program	No. Applications Reviewed	Requested		Recommended	
		Pre	Post	Pre	Post
IAIDP	10	37	40	18	32
MIDP	15	20	61	14	51
Totals	25	57	101	32	83

Table III reflects the estimated number of FTE's for Fiscal Years '81, '82 and '83. It should be noted that the Short-Term Training Grant (T35) awarded to Northwestern University will receive no funds from FY '81 since they were awarded late and the institution had to recruit trainees. The initial review group recommended 28 trainees. To date only one has been recruited.

Table III
Estimated Fellowships and Training (FTE's) Awarded

Postdoctoral	1981	1982	1983
F32	105	115	118
T32	229	222	225
Subtotal	334	337	343
Predoctoral			
T32	145	135	128
Total FTE	479	472	471
T35 (Short-Term trainees)	0	28 trainees	32 trainees
Training Grants			
FTE's 1/	374	357	361
T32	70	59	50
T35	0*	1	1

*Late award made to Northwestern University - recruiting now going on

D. Manpower Survey Conducted by the American Society for Microbiology

An addendum to the 1979 membership survey of the ASM has been prepared in answer to the following questions posed by NIAID:

1. How many Ph.D. and M.D. microbiologists are employed in research in academia and in government, and what is their current specialty?

2. a) What is the complete profile of minority ASM members?
   b) How many minority female Ph.D. and M.D. - ASM members are there? In which sectors of employment and specialty fields do they work?

3. What is the geographic distribution by specialty of respondents of the survey who received NIH support? Which Institutes supported them?

1/ FTE for trainees on T35 not included

4. What degrees were held by respondents who claimed NIAID support?

5. Which recipients of fiscal year 1979 support are ASM members?

The ASM addendum report provides data in answer to these questions.

Tabular data reflects the distribution of Ph.D. respondents by employment sector, work activity and current scientific specialty area. This information should prove valuable to program staff for the purpose of programming and planning.

Another table, "Distribution of Respondents Reporting Federal Support by Region and By Supporting Agency - Summary", compares the NSF, NIGMS, NCI, NIDR, and other NIH components with NIAID. NCI shows the largest number (599) of ASM members as grantees compared with 530 members being supported by NIAID. Although the microbiology population is fairly well distributed according to region it should be noted the distribution for researchers may not be the same. The survey shows that the ASM represents a significant cross section of NIAID grantees and the microbiology grantee population of NIH. The addendum coupled with the original survey data should be very useful in planning for training.

E. Committee Management

The departure of the Committee Management Assistant in June 1981 has left this office with one temporary clerical person until the vacancy can be filled. (See Personnel) The duties of the office have continued uninterrupted with the assistance of several members of the EAP staff. The support supplied by this office for the advisory activities of the Institute have been very successful. Council and Committee appointments have been made on schedule; however, because of the hiring freeze, appointments are being held up in the Office of the Secretary, HHS. With the "new management" in EAP, some streamlining of activities involving Council preparation have resulted in smoother operations. (Attendance at Council meetings have recently been limited to key staff.) Minor irritations on the part of selected Council members have been provoked by the recent dictum to use contract airlines; hopefully we can work out acceptable solutions. The Chairman's Grants (which pay for the expenses of reviews conducted by the MIDAC, AIRC and TBIC) is under close Department scrutiny and efforts are continuing to assure strict adherence to policies governing use of this mechanism.

F. Conference Proposals

Table III shows the conference proposals reviewed by NIAID in FY 1981. The level of funding for conferences in Fiscal Year 1981 (Table IV) was about half of what it had been for each of the three previous fiscal years.

February 1, 1981, the receipt and assignment of all proposals for the support of scientific meetings was transferred from the Fogarty International Center (FIC) to the Division of Research Grants (DRG). All applications for partial support of scientific meetings are now made on grant application form PHS 398 and submitted to DRG according to the same receipt schedule as regular research grant applications. Applications are given an R13 designation and assigned to one or more BIDs using the DRG Referral Guide.

The primary or lead Institute is responsible for the initial review. Applications with a primary NIAID assignment will, as a general rule, be assigned to one of the NIAID chartered committees (MIDAC, TBIC or AIRC). Summary statements will be reviewed by the National Advisory Allergy and Infectious Diseases Council at their regularly scheduled meetings. Following review by the Council, funding determinations on approved applications (both solely and multiple-Institute assigned) will be made by the Research Contracts Advisory Group (RCAG). As with initial review, NIAID will administer the grant on all multiply-assigned R13s approved for funding in which we are the primary or lead Institute.

TABLE III

NUMBER OF CONFERENCE/WORKSHOP PROSPECTI REVIEWED BY NIAID, FY 1981

	R13	Other	Total
Approved for Funding	4	18	22
Sponsorship only	0	0	0
Approved (no funds)	6	0	6
Disapproved or Approved at unfundable priority	1	12	13
TOTAL	11	30	41

TABLE IV

FUNDED OR SCHEDULED FOR FY 1981 FUNDING

	IAIDP		MIDP		Other NIAID		TOTAL	
	#	$	#	$	#	$	#	$
R13 Grant Applications.........	2	$24,000	2	$25,791	0	0	4	$49,791
Other Extramural Prospecti............	6	$18,500	6	$41,200	6	$16,000	18	$75,700
MAXIMUM TOTAL...	8	$42,500	8	$66,991	6	$16,000	22	$125,491

I. CONTRACT MANAGEMENT BRANCH

The Contract Management Branch provides management services to the Institute's Research Program including solicitation, negotiation, award, and administration of all Institute research contracts.

The CMB continues to implement contract policies and procedures promulgated by higher procurement authority. The CMB works very closely with the Program and Project Review Branch and the various NIAID Project Officers and provides contract management expertise necessary in order to continue to have an effective contract program.

Through effective contract management, NIAID has continued to increase its percentage of competitive awards from 26.2% in FY '78 and 37.3% in FY '79, to 60% in FY '80 and an estimate of 67% in FY '81. NIAID is one of the few Institutes which exceeded the competitive goals established by Division of Contracts and Grants.

Other accomplishments for FY '81 are:

1. Led all Institutes in competitive and scheduling goals for FY 80/81 which are established by PHS.
2. Even distribution of contract awards within the fiscal year.
3. Made contract awards in all three socio-economic programs; 8(a), Small Business Administration and women-owned organizations.
4. Led all Institutes (in total percentage) in meeting the goals established by Division of Contracts and Grants in closing-out of contracts.
5. Conducted a seminar for NIAID Project Officers entitled "Research Contracting's Role in Socio-economic Program Compliance."

In FY '81, CMB received approval for 21 contract requests and issued about 805 requests for proposals to organizations which expressed interest. In response to the above advertisements, 44 proposals were received and 14 new competitive contract awards were made.

Due to the requirements to stabilize Research Project Grants (R01s) and the reduction in the NIAID approved FY 81 contract budget, the contract budget has been reduced from $15,254,000 in FY 80 to an estimated amount of $10,662,000 in FY 81. The number of active contracts has been reduced from 160 in FY 80 to an estimated 140 in FY 81. To accomplish this reduction, various funding mechanisms were implemented which included delayed and short-term funding on various research contracts.

II. GRANTS MANAGEMENT BRANCH

Fiscal Year 1981 was a year that continued to reflect the increase in grant activity for NIH, NIAID, and for the GMB. In the NIAID, Grants represent approximately 72% of the Institute's total budget, with over 1,800 grants for approximately $168 million awarded in FY '81. The GMB is responsible for the fiscal and administrative management of all grants and awards issued by the NIAID. The GMB works very closely with the programmatic divisions and provides the fiscal and administrative expertise necessary for effective program management. The GMB also serves as an interpreter of grant policy and procedure issued by the several echelons within the DHHS.

In reference to changes cited in the 1979/1980 reports, we regret to say that: 1) The loss of the categorical Report of Expenditures, which was to be replaced by the non-categorical Financial Status Report, has in fact materialized to the extent that categorical ROE's now represent fewer than one-half the total number of reports submitted. The loss of the categorical expenditures report deprives GMB of a significantly valuable management tool for monitoring grants. 2) That "savings" resulting from the recent change in the project-period concept which enables the awarding component to use the total unexpended balance from one project-period segment as a funding offset to another, will probably exceed $350,000 for FY '81. This brings the two-year savings near the $600,000 level.

Once again, albeit for different reasons, the NRSA award process was delayed and fraught with additional administrative burdens. This year, because of the new administration's budget considerations, we were obliged to make many awards that did not include the customary allowance for "above-the-line" costs and indirect costs. However, subsequent to the issuance of the lesser awards, a congressional action mandating the restoration of these two items resulted in numerous revised or amended actions.

The staff of GMB continues to make significant contributions as members and chairpersons of policy and procedure work groups, committees and subcommittees at the NIAID and at the NIH. Examples of such activity include: NIAID Clinical Research Sub-Panel; NIH Grants Management Self Appraisal and Training; PHS Grants Policy Statement; NIH Committee on Short-Term Training; NIH Working Groups on questions of "Automatic" Carryover, Intermingling of Funds Between Grants, and Changes in Requirments for Expenditure Reporting. Additionally, the Chief, GMB, participated as a faculty member at seminars where grantee personnel were in attendance, such as the Seminar on Administration of NIH Grant Programs, at the University of Washington, Seattle, which was held for the northwest region of the U.S. The Chief, GMB, was also a faculty member for numerous PHS and NIH training courses attended by PHS and NIH personnel.

Further, the Chief, GMB, is currently in the second year of a three-year term as a member of the NIH STEP Committee where he will direct a newly-formed module, "The Shrinking Research Dollar: Funding Issues, Mechanisms, and Alternatives."

In keeping with the Branch philosophy, employees were encouraged to broaden their horizons beyond their day-to-day routine and fill spots created by temporary absences. Classes and seminars were attended with more regularity. Special emphasis was placed on furthering the partnership role between the grants management specialists and our program administrator counterparts. Project site visits were attended where practicable. In general the staff of GMB continued to move forward in their individual development while maintaining a high level of excellence in pursuit of the day-to-day duties and responsibilities that encompass all grant programs funded by the NIAID.

III. PROGRAM ANALYSIS AND EVALUATION BRANCH

The Branch is responsible for the official NIAID scientific and administrative management information system used to develop reports, to respond to data and information requests, and to support the Office of the Director, NIAID Program Directors and the NIAID staff. The Branch also serves as a focal point for management and budgetary data for the Extramural programs; referral liaison with the Division of Research Grants; NIAID ADP Security Compliance coordination; ADP liaison with other Institutes and NIH; and reporting Trans-NIH and other health portfolios.

A management data base (Program and Financial data) has been developed by Branch personnel and validated by program staff. All competing grants applications, non-competing grants, contracts, and intramural projects have been entered into the Multi-Axis Coding system, (MACS). For FY 1980, 2,954 separate entries are in the validated MACS data base. FY 1981 has 2,173 validated entries not including FY '81 Intramural Activities.

Data elements included in the MACS data base identify Clinical Trial Percentages; Research Objectives; Special Research Portfolios; Trans-NIH areas, Basic, Applied or Developmental Research Areas for each grant, contract and intramural project.

In order to produce more timely and accurate reports and enhance data credibility and consistency, a procedure for the control and documentation of the management data base has been developed by PAEB. Health Science Administrators review, after Council, printouts of all competing applications for the MACS codes assigned by PAS staff to projects in their programs. Any modifications are submitted to PAEB.

For the first time, the NIAID portion of the HHS ADP Systems Security Policy Compliance Report has been submitted quarterly.

A. Fiscal Management and Analysis Section

The FMAS is responsible for research and training grant budget projections, development of operating budgets, timing of grant payments, and fiscal analysis and reporting for the Extramural Activities Programs.

The operating budget for traditional research grants in FY '81 increased by 11.7%. In spite of this modest increase in dollars, there was a 6% decrease in the number of competing research grants which could be paid this year due to accelerating indirect costs and inflation.

The basis for payment of research grants changed in FY '81 from normalized scores to raw priority scores. Grants with a priority of 178 or better were paid this year. In addition, 31 applications with scores beyond the 178 cut-off level which had special relevance to the programs of the NIAID, were identified by staff, approved for funding by Council, and paid. The pay rate for traditional research in FY '81 was 33%.

The number of competing research career and academic awards made in FY '81 fell dramatically, from 25 in FY '80 to just 5 this year, reflecting a significant drop in the budget for this mechanism.

Payment of all individual fellowship awards and training grants was held up mid year for several months while NIH developed policy regarding payment of indirect costs and institutional support on these mechanisms. With the final decision to pay full indirect costs and allowances in FY '81 coupled with a reduced training budget, the number of FTEs was reduced over 8%, from 522 last year to 479 this year.

B. Data Control Section

The functions of the Data Control Section, using the NIH computer facilities, include generating management, budgetary and programmatic reports for the Extramural Activities Program, the Immunology, Allergic and Immunologic Diseases Program, and the Microbiology and Infectious Diseases Program, as well as other areas of NIAID such as the Office of Program Planning and Evaluation, Information Office, Budget Office, and Office of the Director in the areas of grants, contracts and Intramural activities.

The 1980 Awards and Projects Activities Book was prepared using the Multi-Axis Code System, (MACS) and the NIAID management information data system. All areas of NIAID support were identified by MACS, including intramural projects, and extramural grants and contracts. Standardized files and reporting the formats were developed to retrieve data of active and funded projects using one rather than three or more retrieval programs.

Data Control Section has been participating with NIH-OD Personnel in a management review of NIAID registered data systems.

All competing applications coded by the Program Analysis Section have been entered into the MACS and a formatted description of each application has been distributed to the appropriate program administrator and portfolio officer for validation. The total FY '81 data base, excluding changes in intramural projects, has been validated.

C. Program Analysis Section

During the past year, the Program Analysis Section (PAS) has combined the utilization of the MACS with the validation and standardization of the data within the system. In FY 1981, PAS classified the scientific and programmatic content of approximately 754 FY '81 competing applications through 190 priority score. This information was subsequently incorporated by the Data Control Section with existing data to produce a data base of approximately 2200 active grants for FY 1981. This was accomplished with a staff reduced to one half. All 2154 incoming applications were assigned to appropriate program administrators. PAS staff is continuing to sharpen Program descriptions of content, to develop selection criteria, and to standardize request formats so that NIAID's data collection system for handling scientific and programmatic management information is both flexible and responsive.

An increase of interest in the *Trans-NIH Health Research Problems and Similar Portfolios* by the Administration, members of Congress, and the general public has placed upon PAEB the responsibility of insuring complete and comparable data when reporting these activities: Arthritis; Blood Related Diseases; Bronchopulmonary Disorders; Cerebral Palsy; Cystic Fibrosis; Diabetes; Digestive Diseases, Dermatology; Epidemiology; Genetic Diseases; Hepatitis; International Health Research; Kidney Diseases; Lupus; Nutrition; Prevention; Recombinant DNA: Reye's Syndrome; SIDS; Toxicology; STD; Immunology and Virology.

IV. PROGRAM AND PROJECT REVIEW BRANCH

A. Introduction

As in the past, the Branch continued to have a busy schedule. The reduction in the MIDAC review workload because of fewer RFA's and RFP's was compensated for by an increased number of program evaluations. This was the last year the AIRC and TBIC would be identified as separate committees, having been combined as of July 1 into one, the Allergy, Immunology and Transplantation Research Committee with two subcommittees. However,, for all practical purposes, the AIRC and TBIC subcommittees will work independently as before, meeting once a year as a full committee. Still another review function has been added to the Committees, this time the review of conference (R13) applications. The departure of the MIDAC executive secretary, Dr. Thelma Fisher, and her replacement by Dr. Susan Spring, formerly of Dr. Chanock's laboratory has thankfully not created any discernible disruption of work involving that committee. A replacement for the other executive secretary, Dr. Harley Sheffield, is actively underway. Orientation of new MIDAC members and liaison members was conducted.

B. Microbiology and Infectious Diseases Advisory Committee

The Microbiology and Infectious Disease Advisory Committee held four meetings: October and December 1980 and March and July 1981.

Grants: The MIDAC reviewed one program project renewal plus one supplement to a program project and 21 institutional fellowship applications, all of which were approved. The total dollars reviewed for grants were $2,792,241 and the total dollars approved were $2,088,397.

Contracts: The full MIDAC reviewed 22 contract proposals involving 7 RFP's, 4 noncompetitive renewals and one unsolicited proposal. Fourteen proposals were approved and seven awarded. The total dollars competitively reviewed by MIDAC for contracts was $3,607,957 and the total amount awarded was $1,701,126. The three proposals renewed non-competitively were all approved. One unsolicited proposal was reviewed and approved, but not awarded. Three ad hoc meetings were conducted to review 5 contract proposals to 3 RFP's. All three were for new and competing proposals. Four of the proposals were approved, but only one proposal was awarded.

At the December meeting the MIDAC reviewed and approved a U.S. Army gonococcal vaccine development project for the Walter Reed Army Institute of Research.

Program Evaluation: The Enteric Disease Program was evaluated at the October Meeting. At the December meeting, the committee made a recommendation that the Institute make efforts toward the characterization of transfer factor and the development of standardized methods for its production and evaluation. At the July meeting, the committee evaluated the Animal Models for Antiviral Evaluation Program. Details of these program evaluations should be found in the MIDP annual report.

C. Allergy and Clinical Immunology Research Committee

The AIRC met three times during the period of October 1980 through September 1981, reviewing 16 program projects and centers, 9 institutional fellowship and 3 Allergic Diseases Academic Award applications, of which 16, 9 and 1, respectively, were approved. The total dollars reviewed were $6,725,383 and the total dollars approved were $4,532,086. The program projects and center applications reviewed were in the areas of Asthma and Allergic Diseases and Mechanisms of Immunologic Diseases. Thirteen were site visited. Project site visits are underway for program project applications in response to the RFA: Mechanisms of Immunologic Diseases, that will be reivewed by the AIRC at its October 1981 meeting.

Four contract proposals totaling $2,463,482 were reviewed and one was approved for $608,790. All four were received in response to RFP 81-8 "Allergen Resource Reference Library."

Program Advice: Program staff sought the Committee's advice on a variety of issues including food allergy, allergen reference reagents, and transfer factor studies.

D. Transplantation Biology and Immunology Committee

The Transplantation Biology and Immunology Committee met four times in FY 1981, reviewing two institutional fellowship applications and approving both. One program project and one center grant application were reviewed and approved. Nine conference proposals were reviewed and approved. Total grant application dollars reviewed were $513,702 and $402,824 were approved. One proposal submitted in response to RFP-82-2, "Development of Methods to Prepare Bulk Supplies of Alloantisera Specific for Murine Cell Surface Determinants" was reviewed and approved. Dollars requested and approved were $109,279. An interagency agreement requesting $130,309 was reviewed, deferred, site visited and rereviewed then eventually disapproved.

Program Advice: Program staff sought advice from the TBIC on a variety of issues including the KTHS program, the serum bank, HLA tissue typing trays and homozygous typing cells.

Concept Clearance: The Committee cleared the concepts of a rapid cellular cross-matching test, characterization of ethnic antigens, and acquisition of normal human lymphoid cell lines for distribution.

E. Review Services Unit

The Unit continues to be understaffed and the position of Head of the Unit has not yet been filled since October, 1980. After considerable negotiation with the Personnel Office, the position was finally restored to its original GS-7 level. A QRB is currently underway for this position. We were very fortunate in having Ms. Barbara Wilson, detailed to us from NIA, who skillfully put organization and order to the Unit. Unfortunately, we cannot retain Ms. Wilson who will transfer to the DFM where promotion possibilities are greater for her. Because of the seriousness of the personnel shortage and a combination of desperation and imagination, the Acting Director and Acting Deputy Director of EAP decided with the concurrence of the Director, NIAID, to drastically reduce the number of "Council Books" (Books routinely prepared by RSU staff composed of all summary statements for Council review) prepared for Council and staff. The experiment was successful in that no complaints were received from Council nor staff and, on the contrary, operations were smoother than before. Ms. Wilson will be sorely missed but we hope the stage has been set and the momentum will continue for smoother Council preparations.

F. Clinical Trials

The capability of NIAID to maintain and monitor its investment in clinical trials continues to be an essential function of the Extramural Activities Program. Due to a reduction in DRG staff responsible for the annual collection of data for the NIH Inventory of Clinical Trials, receipt of the FY 1980 inventory questionnaires has been indefinitely delayed. However, information on the clinical trials supported by NIAID grant, contract, and intramural mechanisms in FY 1980 have been collected by the Inventory coordinating staff. Analysis of this data has been used for a variety of internal purposes and to respond to Congressional and Department inquiries. (The NIH investment in clinical trials has been a recurring topic of discussion at the Congressional Appropriations Hearings in recent years.) In addition, the Inventory is an integral part of the SATT classification of NIH activities and is used as a basis for identifying the clinical trials element in other reporting systems and in the annual report to the NSF.

A total of 112 clinical trials were reported for FY 1980: 53 grants (77 trials), 23 contracts (29 trials) and 3 intramural projects (6 trials) for an estimated amount of $5,496,979. The number of trials initiated in FY 1980 decreased approximately 36% from FY 1979; the cost of new trials decreased by approximately 46%. The total number of trials in FY 1980 increased by 3; the total cost of clinical trials supported by NIAID in FY 1980 decreased by approximately 15%. This difference was caused by the decrease in number and dollars of clinical trials being supported by the contract mechanism. (See Table V)

TABLE V
NIAID CLINICAL TRIALS
FY 1979 - 1980

	FY 1979 No. of Projects	FY 1979 No. of Trials	FY 1979 Amount	FY 1980 No. of Projects	FY 1980 No. of Trials	FY 1980 Amount
Ongoing Trials						
Grants	36	55	$1,751,359	45	69	$2,565,914
Contracts	10	23	2,034,454	14	16	1,321,225
Intramural	3	6	234,000*	3	6	307,500**
Subtotal	49	84	4,019,813	62	91	4,194,639
New Trials						
Grants	13	15	767,727	8	8	132,652
Contracts	13	10	1,665,335	9	13	1,169,688
Intramural	-	-	-	-	-	-
Subtotal	26	25	2,433,062	17	21	1,302,340
Total Trials						
Grants	49	70	2,519,086	53	77	2,698,566
Contracts	23	33	3,699,789	23	29	2,490,913
Intramural	3	6	234,000*	3	6	307,500**
Total	75	109	$6,452,875	79	112	$5,496,979

* 3.6 Man Years x $65,000
** 4.1 Man Years x $75,000

V. RESEARCH RESOURCES BRANCH

A. Special Activities

Mrs. Sylvia Cunningham continued to serve as Acting Branch Chief during this period. In addition to the Chief, the RRB is supported by one clerical position. The RRB is responsible for the management of operations including distribution and cataloging of the microbial, allergen and some of the immunologic reagents.

The need for well-characterized reference reagents as an adjunct to research is well recognized, and in support of the concept, the RRB conducts a program which distributes the reagents with information and technical advice on their characteristics and uses.

This year the American Society for Microbiology (ASM) changed the date of their annual meeting from May to March. Since only 10 months had elapsed since the previous NIAID exhibit was shown at ASM and because the exhibit had not been updated, a decision was made not to exhibit at the ASM meeting held in March in Dallas, Texas. The Branch does plan to make arrangements for an exhibit at next year's ASM meeting to be held in Atlanta, Georgia.

B. Asthma and Allergic Diseases Program

Under a procurement contract with the American Type Culture Collection, the RRB has been responsible for the processing and packaging of additional lots of penicillin minor determinant products which are being used by six NIAID contractors to perform clinical studies to ascertain the penicillin sensitivity of patients. The Branch has made the arrangments to ship the contractors the necessary materials as needed.

The Branch also arranged for the processing and packaging of 10 lots of hypersensitivity antigens and antisera. This material has been sent to the certification laboratory for final ampoule testing. Arrangements are now being made to process and package short ragweed extract and an antibody to the extract which will be offered to WHO as a reference preparation. This project is scheduled for completion during the summer.

A cat pelt extract was received from Hollister-Stier. The material has been cataloged and is now being tested by an independent laboratory.

In addition to the activities listed above, the RRB has continued to distribute allergenic products such as ragweed antigen E, K and Ra3, ryegrass I, II and III and venoms of honey bees, yellow hornets, white-faced hornets, yellow jackets and hymenoptera venom diluent; these venom products are available for distribution under an IND as well as for in vitro use.

C. Immunological Reagents and Resources

The contract awarded in FY 1978 to Flow Laboratories for the maintenance and breeding of rabbits of known genotype for use in immunological studies, has continued to serve a useful purpose in maintaining a colony of 500 specially-bred rabbits for use in NIAID intramural immunological studies. In addition, these facilities provide reference quantities of reagents and limited numbers of rabbits to responsible investigators doing immunological studies. During this period, 23 shipments of rabbit antisera have been made to investigators. Due to the limited amount of funds available for contract purposes, the Institute is pursuing the possibility of merging this project with a similar one sponsored by the Laboratory of Immunogenetics. Site visits are planned to both locations (Flow Laboratories and Spring Valley Labs) to determine the best way to handle such a merger.

D. Microbiology

During this period the contract with the University of Alabama for developmental work to determine optimal conditions for the preservation, retrieval, storage and distribution of fragile bacterial host strains was completed. Three basic methods of storing were studied, i.e., 1) preservation in paraffin-sealed agar stabs, 2) by freezing in peptone-glycerol broth and 3) by lyophilization. It has been determined that the optimal preservation method is lyophilization. However, lyophilized cultures and freshly-prepared agar stabs of chi 1776 and several other E. Coli K-12 strains survived almost equally well during mailing experiments lasting five to sixty-three days at ambient temperature. A set of General Specifications for Packaging, Maintaining and Distributing Bacterial Host Strains and Bacteriophage Vectors for Recombinant DNA Research was submitted as part of the final report of this contract. These specifications will be followed in any future projects.

The NIH subtype-specific antisera to hepatitis B surface antigen were finally accepted as WHO reference standards. Two-hundred and fifty ampoules of each of the three subtypes were shipped in January 1981 to the WHO Hepatitis Laboratory located in the Netherlands for storage and distribution.

Branch staff participated in the preparation of a contract request for additional arbovirus-reference reagents. If adequate funds are available, a contract will be awarded this year for the production of from three (3) to ten (10) new arbovirus reagents. Upon completion these new reagents will be packaged, labelled, cataloged and made available for distribution by this Branch.

E. Research Resources - Research Reagents

While responsibility for the actual production of reagents is with the various Institute program areas, RRB has continued to sponsor certain service-type contracts which serve the needs of the various program elements.

Upon notification of any problem from investigators using any item distributed by this office, arrangements are made to have the product tested by a competent laboratory. This year a report was received that the NIAID respiratory syncytial virus - strain 18537 could not be detected when a sample was used by an investigator. Arrangements were made to have this product tested by the Molecular Anatomy Program, Georgetown University. Confirmation of the investigator's report was made. Apparently the mycoplasma contaminant (known to exist at the time this item was listed in the catalog) has continued to grow and has overpowered the virus thus making it undetectable. At this time there are no plans to have a new batch prepared.

After a category of reagents has become established, and the area of research that it supports is well defined, it is of questionable value for RRB to continue storing and distributing that category of reagents for a prolonged period of time. During FY 1975, it was determined that four viral reagent groups fall into this category. The RRB therefore, entered into a five-year contractual arrangement with the American Type Culture Collection (ATCC) to transfer the enterovirus, adenovirus, rhinoviruses and arboviruses reagent collections to the ATCC whose prime function is to store and distribute these types of reagents.

The assaying of reagents for all four viral groups has been completed and transfer of all reagents to ATCC has been made. The ATCC is now totally responsible for distributing these reagents. The Branch does make arrangements for NIH laboratories to receive these materials at no cost. All other investigators (both US and foreign) are required to pay ATCC a handling fee (somewhat reduced from ATCC's normal fee) and shipping costs.

The RRB arranged for the labeling and packaging of the virus and guinea pig serum for rotavirus type 2 Wa (Ma 104 - grown). This item has been cataloged and is now available for distribution. A patent application has been made for the item.

The intensive work of prior years has resulted in the completion of work on the enterovirus, adenovirus, rhinovirus, myxovirus and the agents and antigens of hepatitis A and B. In most cases, seed virus preparations and corresponding antisera are now available for most of the viruses of public health interest. The reagents which have resulted from the various projects have been most useful, particularly the hemagglutinins, neuraminidases, ribonucleoproteins and the influenza viruses of man and animals.

F. Processing and Distribution

In addition to the various program elements detailed above, the RRB also distributes coronaviruses, herpes viruses, interferons, mycoplasmas and reoviruses.

Due to the limited number of reagents to be processed during FY 1981, there was not a formal research and development contract to handle this activity. However, six procurement contracts were made to the American Type Culture Collection to process the penicillin minor determinent mixture, short ragweed, rotavirus type 2, and the hypersensitivity pneumonitis reagents.

The RRB reagent collection now consists of over 350 individual reagents. The repository and distribution contract remains at Flow Laboratories, McLean, Virginia. A tabular record of distribution by the facility since FY 1973 follows.

DISTRIBUTION OF VIRAL, MYCOPLASMAL, AND ALLERGEN REAGENTS

Fiscal Year	Total Transactions	Total ampoules & vials distributed
1973	575	19,181
1974	500	9,932
1975	592	6,751
1976	762	10,188
1976 (TQ)	192	3,126
1977	613	7,633
1978	605	6,851
1979	782	16,223
1980	793	11,238
1981 (3/4 year)	668	8,784

In addition to the shipments tabulated above, Flow has also made 20 shipments for the Development and Applications Branch, 23 shipments of rabbit sera, 1 interferon shipment for the National Cancer Institute and 32 shipments for the Epidemiology and Biometry Branch.

OFFICE OF THE SCIENTIFIC DIRECTOR, NIAID

1981 Annual Report

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SUMMARY OF PROGRAM

Laboratory and Clinical Research, NIAID

October 1, 1980 through October 1, 1981

Office of the Scientific Director

The Annual Report of the Intramural Research Program contains individual summaries of research projects in the twelve laboratories which constitute the research component of the National Institute of Allergy and Infectious Diseases. Administrative responsibility for the Intramural Research Program resides in the Office of the Scientific Director (OSD). The administrative functions of OSD remain the responsibility of Mr. Charles Criswell at the Bethesda campus and Mr. Robert Steiner at the Rocky Mountain Laboratory. Special assistants in the OSD include Dr. Gordon Wallace who has major responsibility for intramural contracts, Dr. Katherine Cook Jaouni who maintains responsibility for EEO and safety activities of the Intramural Program, and Dr. Herbert Stoenner who serves as special assistant at the Rocky Mountain Laboratories.

Recruitment of personnel has been a particularly difficult problem during this year. The Department of Health and Human Services instituted a personnel freeze in March of 1980 which has been extended to the present time. Because of the freeze, it has not been possible to recruit a Chief for the new Laboratory of Immunopathology at the Rocky Mountain Laboratory, which is scheduled to replace the Epidemiology Branch which is currently headed by Acting Chief, Dr. Robert Philip. Communications between the Bethesda campus and the Rocky Mountain Laboratory have continued to improve. National and international meetings have been supported at RML,including a meeting on Bacterial Virulence held in the summer of 1981. A major renovation project was undertaken to develop a centralized electron microscopy facility at RML. This modern laboratory has the capacity to house up to four electron or scanning microscopes and will be directed by Dr. Claude Garon who has transferred from the Laboratory of Biology of Viruses on the Bethesda campus to the Rocky Mountain Laboratories where he will serve as the first Chief of the Electron Microscopy Section. Renovation of buildings and spaces at RML continue to be necessary as recruitment of junior scientists proceeds despite the freeze. Mr. Robert Steiner, as well as members of the RML shop services, continue to perform an outstanding job in the renovation of the laboratory facilities.

The Editorial Office of the Intramural Program has been extremely active. The chief editor, Betty Sylvester, has not only provided a means for highly professional editing of manuscripts in the Intramural Program but also has been involved in the completion of manuscripts which have been published as books, including a book on "The Lymphocyte" and a book on "Mucosal Immunity". Major assistance has been provided by Dr. Ruth Guyer who has undertaken the extensive task of scientific editing of the chapters of these books. The IBM text editing machines have been updated this year by their exchange for a centralized, computer facility which expands the versatilization and capability of the Editorial Office. The quality of the publications has been particularly notable leading to compliments not only from intramural scientists but also from the Editorial Staff of several of the Journals who receive the scientific manuscripts.

For the third successive year we have held a Biomedical Science Program, during which over fifty students were invited to NIH to spend three days with scientists in their laboratories, learning of work opportunities at NIAID as well as other Institutes of NIH. Dr. Katherine Cook Jaouni provided the leadership and management of this program. The freeze on personnel caused some difficulty as the number of summer students had to be reduced drastically because of ceilings placed on NIH by the Department of HHS, thus only 34 summer students could be placed which represent approximately one-half of those in the previous years' summer program. Students involved in the Stay-In-School Program at NIAID were, of course, allowed to work full-time in the summer so that the summer program totaled 34 students of whom 14 were female; of these 1 was a native American, 5 were Asian, 8 were Black and 5 were Hispanic. We continue to have a shortage of Hispanic and native American employees at all grade levels. There is also a shortage of all minorities and women in Grade GS-13 and above. At Grades GS-11 and below, minority participation is equal to or greater than the number which represents the percent of minorities in this community. The solution to our hiring problem is of two varieties - those personnel in GS-13 and above are most commonly research scientists, and the recruitment must be undertaken in professional schools using the same criteria for quality that we have expected for our science program at NIAID. The success of our summer program (even though limited) would seem to indicate that we still are on the path to identifying minorities and women candidates for our post-doctoral and scientific positions in years to come. Cooperation with the Hispanic coordinator in the Division of Equal Opportunity at NIH may assist in the recruitment of Hispanic and native American employees at grade levels below GS-13.

We continue to progress in the development of animal care facilities. During the next fiscal year, we will have completed the renovation of facilities to such a degree that reexamination by AAALAC should result in full accreditation. A breeding contract which was established to perform in full compliance with AAALAC standards has begun to provide congenic strains of mice. The holding contract at Litton is gradually being phased out due to shortage of funds. Dr. Peter Golway serves as the senior veterinarian for OSD and has provided outstanding support for the development of the Veterinary Services Program. He has been ably assisted by Mr. Carter Smith. A shortage of funds will require a close look at all animal care contracts, and it is anticipated that some contracts, such as those for rabbit care, must be consolidated to provide savings on administrative costs.

The Intramural Program of NIAID continues to have a major commitment to the Risk Assessment Program for Recombinant DNA Research. Dr. Malcolm Martin has completed his study of the murine leukemia plasmid- E. coli experiments to explore viral pathogens. Dr. Martin has also worked with members of the NIH Safety Branch to develop protocols for the study of cloning experiments using vector transmission into wild-type E. coli under P4 conditions. This work was to be conducted in a newly-constructed P4 facility on the NIH campus (Bldg. 41-T). Unfortunately, this building has not yet met P4 test conditions. Dr. Martin has also discussed and made available the resources of the Intramural Program for an extramural (Harvard) experiment involving tetanus, toxin gene clones in E. coli, utilizing the P4 laboratories of Building 550 at the FCRC.

The "Round-Robin" Renovation of older buildings on the NIH campus is continuing to be implemented even though Congressional appropriation and OMB review have failed to support the inclusion of funds for the renovation of Building 8. Nevertheless, the renovation of spaces in Building 5 and the transfer of animals from Building 5 to Building 14B (veterinary Resources Branch) continues on schedule. Authorization for renovation of animal spaces and other laboratory spaces in Building 5 was obtained after extensive architectural review of Building 5. Work on spaces in Building 5 will commence early in fiscal 1982, and it is expected that personnel in Building 8 will move to Building 5 between January and March of 1982.

The Ambulatory Care Research Facility neared completion this year with occupation by NIAID partially in September of 1981. The ACRF will provide space for an expansion of the Clinical Allergy Research Program and the related program of Clinical Parasitology which also involves immediate hypersensitivity. In support of the Round Robin Renovations, the Deputy Director for Science of NIH provided four additional floors of animal space in the ACRF to the IRP, NIAID, for housing of animals which will be displaced during the initial stages of the move of personnel from Building 8 to Building 5.

Safety continues to have a high priority in IRP/NIAID. A new smoke-detection warning system has been installed in Building 7. Those facilities, located in the sub-terranean basements of Building 7, the old incinerator area, were abandoned this fiscal year because of the danger to personnel due to the restricted egress from these areas.

During this year, one laboratory has been disbanded. The Laboratory of Streptococcal Diseases was disestablished with the impending retirement of its Chief, Dr. Roger Cole. Members of the laboratory have been reassigned, either to the Laboratory of Microbial Structure and Function at the Rocky Mountain Laboratories or the Bacterial Virulence Section of a new Laboratory which was created this year. The new Laboratory, called the Laboratory of Molecular Microbiology, is headed by Dr. Malcolm Martin and will consist of three Sections, two located in Building 5 at the Bethesda campus, and a third, Bacterial Virulence Section, located in Building 550 at the Frederick Cancer Research Center in Frederick, Maryland.

A contract with KOBA Associates has been completed during the year. This work provided for the analysis of capabilities and knowledge of intramural supervisors. KOBA has also provided an evaluation of the intramural administrative and management operation and made suggestions regarding management changes and training opportunities to improve supervisory performance.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00209-01 OSD

PERIOD COVERED
October 1, 1980 to September 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Biology of Mousepox and Development of a Specific Serologic Test for Ectromelia Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Co-PI:	Gordon D. Wallace	Assistant Scientific Director, NIAID
	Pravin N. Bhatt	IPA, OSD, NIAID
Other:	Bernard Moss	Chief, Molecular.Struc.Sec., LBV, NIAID
	Herbert Morse	Chief, Vir.and Cell.Immunology,LMI,NIAID

COOPERATING UNITS (if any)

The Australian National University

LAB/BRANCH
Office of the Scientific Director

SECTION
Office of the Scientific Director

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.0	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Because of recent epizootics of mousepox at NIH and other biomedical research institutions in the U.S., research was undertaken to develop a simple, sensitive and specific test to detect antibody to ectromelia virus, and to develop an assay for infectious virus. In addition, a study of biological and biochemical markers of virus strains from various parts of the world as correlates of epizootiology and pathogenesis of mousepox are in progress. This basic information and diagnostic methodology are considered essential for a rational approach to the control and prevention of mousepox, which threatens serious disruption of biomedical research in the U.S.

Background

During the 1979 outbreak of mousepox at the National Institutes of Health and other research institutes in the U.S., the lack of a sensitive and specific serological assay and lack of information on the biology and epizootiology of ectromelia virus greatly hindered control efforts. Answers to questions on virus strains, such as differences in antigenicity, virulence, and communicability were not available. Moreover, influence of specific mouse and virus strain interaction on pathogenicity, the frequency of the occurrence (if at all) of chronic or latent infection with virus shedding and its effect on the epizootiology were not known. Because in the U.S. the virus has not been enzootic, the emphasis has been on exclusion and scientists have avoided working with it. The resultant lack of information made it very difficult to initiate intelligent and rational control measures. Therefore, a program to study some of these aspects was initiated by IRP, NIAID. Intramural research was chosen over a grant or contract to save time.

The objectives of this research are as follows: (1) Development of a sensitive *in vitro* assay for detection of infectious ectromelia virus, (2) development of a sensitive and specific serologic assay for detection of antibody to ectromelia virus (3) comparative studies of the biology and pathogenesis of virus strains, collected from various parts of the world, in relation to inbred mouse strains, (4) endonuclease mapping of DNA among these strains of viruses. Of particular interest is whether chronic persistent and/or latent infection of the host with chronic shedding of the virus occurs.

Virus Isolation

L and VERO cells were initially evaluated. These cells not only were not as sensitive as mice for virus isolation but also there was much variation in sensitivity between passage levels of cells (cultures were received weekly from BOB). Therefore, based on Dr. Moss' experience with vaccinia virus, we tried BS-C-1 cells, clone F-19 (cloned by Dr. Salzman of LVD, NIAID) and found them to be very susceptible to ectromelia virus. Initial results indicate that sensitivity of these cells in detection of ectromelia virus is very close to that of the mouse. Definitive studies are needed to confirm this observation. Liver and/or spleen samples collected from mouse colonies suspected of being infected with ectromelia were tested for virus. Using both mice and/or tissue culture system, we have isolated virus strains which behave like ectromelia virus from samples received from Washington University (St. Louis), University of Minnesota and Chicago University. These viruses will be further identified. In addition, the following virus strains have been received from Dr. Frank Fenner: Moscow, Hamstead (Both from England), Ishibashi (Japan) Beijing

62-2/70, Beijing No. 5/78 (both from China) and Saint Louis 1969 (France).

Development of ELISA with ectromelia virus as antigen.

Antigens tried initially, partially purified virus from infected tissue cultures (L, Vero and BS-C-1 cells) and similarly prepared control antigen from uninfected cell cultures, have given satisfactory results. Ectromelia virus was inactivated by treatment with psoralen derivative and ultraviolet light. In evaluating specificity, we tested 150 sera from known ectromelia infected and uninfected mice with good results. Specificity was also demonstrated by testing 80 sera collected over a two-year time period from an ectromelia infected colony. Fifty-one sera were positive and 29 were negative by both ELISA and indirect fluorescent antibody (IFA) tests. To date, more than 300 mouse sera have been tested by ELISA with excellent results. In an ELISA test to compare vaccinia and ectromelia antigens, the same 80 sera mentioned above were used in a collaborative study with Microbiological Associates (MA) and Litton Bionetics (LB) (MA and LB used an ELISA with vaccinia antigen). MA's test results agreed with all our positives but they had 6 sera positive which were negative both by ectromelia antigen ELISA and IFA tests. There was complete agreement between our results and those of LB.

In a preliminary study comparing ectromelia and vaccinia antigens, vaccinia immune sera reacted to a significantly higher titer with vaccinia antigen than with ectromelia antigen, but the converse was not observed. Thus, it appears that there is a one way cross between vaccinia and ectromelia and that either antigen is satisfactory to detect antibodies in mice following infection with ectromelia virus. However, ectromelia virus is a poor antigen for detection of antibody to vaccinia.

Studies in mice on the biology of ectromelia virus have been delayed because of frequent mechanical failures in the mobile containment laboratory, and lack of technical help (due to illness). Most of the work was carried out between September 1980 and February 1981. For comparative studies on virulence of NIH-79 and Moscow strains of ectromelia virus, Balb/c Ann (susceptible) and C57BL/6 (resistant) mice were used. Other strains of mice such as Balb/c (Jax) and DBA/2 were also used. Observations can be summarized as follows: a) Balb/c Ann (NIH) is of intermediate susceptibility to mortality following foot-pad inoculation with either strain of virus. Balb/c (Jax) is highly susceptible to mortality following foot-pad inoculation with Moscow strain but not when inoculated with NIH-79 strain, b) C57BL/6 is highly resistant to mortality following foot-pad inoculations with either strain of virus, c) DBA/2 are not as susceptible to mortality as Balb/c (Jax) following inoculation with Moscow strain by foot-pad route. In this respect, they are comparable to Balb/c Ann (NIH).

Results with Saint Louis (France) and Buijing (Japan) strains of viruses in DBA/2 mice were similar to those obtained with the Moscow strain of virus.

Priorities for future work

Evaluation of diagnostic procedures will include comparison between BS-C-1 cells and various strains of mice for virus isolation. Further evaluation of the ELISA test will include testing of large numbers of sera from conventional mouse colonies free of ectromelia virus. Also, comparisons between ELISA and IFA test will be expanded. Plaque reduction neutralization and hemagglutination inhibition test also will be compared with ELISA test. Further comparisons of homologus and heterologus reactivity between vaccinia and ectromelia antigens and antibodies will be carried out by ELISA and IFA test. Hopefully, this will provide tests to distinguish between infections by these two viruses. Sequential antibody development in mice to both ectromelia and vaccinia virus will be monitored by ELISA and, if possible, by IFA test.

Biochemical studies will be continued to compare viral DNA from various strains of ectromelia virus by endonuclease restriction mapping. If clear-cut differences between ectromelia virus strains can be detected, this method, then could be used as an epidemiological tool to trace sources of ectromelia virus in mousepox outbreaks and to compare virulence of virus strains.

In biologic studies it will be important to determine whether ectromelia virus persists in the mouse host. It may be necessary to use a variety of mouse strains and selected virus strains for this purpose. In collaboration with Dr. Morse we will attempt to map the resistance gene location in C57BL/6 mice. It will be necessary to determine susceptibility and resistance of Balb/c (Bailey) and C57BL/6 (Bailey) mouse strains to ectromelia virus. If we can demonstrate a clear-cut difference, then further experiments will be performed with appropriate congenic mice.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00214-01 OSD

PERIOD COVERED
October 1, 1980 to September 1981

TITLE OF PROJECT (80 characters or less)
Epizootiology of Mousepox

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)
Research Diagnostic and Investigative Laboratory, College of Veterinary Medicine, University of Missouri; St. Louis University, Medical School; Washington University, St. Louis, Mo.

LAB/BRANCH
Office of the Scientific Director

SECTION

INSTITUTE AND LOCATION
NIAID, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An epizootic of mousepox at the National Institutes of Health in late 1979 (preceeding outbreaks in biomedical research institutions in four other states) caused severe disruptions in research and revealed a serious deficiency in knowledge of the epizootiology and biology of this disease. Since such information is essential to developing effective strategies for prevention and control, epizootiologic studies were commenced (in April of 1980). A computer-based system was developed for the collection and processing of data, which included calculations of prevalence of cases by time, place, mouse strain, etc. Data from the NIH outbreak and outbreaks at other institutions indicated that the virus behaves differently in different inbred strains of mice. At NIH as well as elsewhere, infected mice had been present for several months before recognition, providing potential for extensive spread of the virus. However, virus remained localized within a few cages in a few rooms. Nevertheless, due to misconceptions of the epizootiology of ectromelia virus in inbred mice and nonspecific results from serologic assays, large numbers of mice were unnecessarily destroyed.

PHS-6040
(Rev. 10-76)

In November of 1979, mouse pox was diagnosed in a small group of sick mice that had been submitted for diagnostic purposes early in October. These mice had been housed in a mouse colony in an NCI immunology laboratory. It became apparent immediately that mice infected with ectromelia virus had been in this room for at least three months, if not longer, and during this period mice from the same colony had been provided to investigators in other laboratories at NIH, and held at NIH contract facilities outside of NIH. Because of the potential seriousness of the situation, all mouse rooms at NIH, as well as related contract facilities, were put under quarantine and a surveillance for ectromelia virus was begun. Ultimately at least one mouse in every cage of mice on campus and at related contract facilities were tested for antibody to ectromelia virus, approximately 20,000 mice. The cost of the epizootic, including man hours in investigation and surveillance, diagnostic tests and destruction of mice approached $1,000,000. The major difficulties in contending with this outbreak was the absence of a sensitive and specific serologic assay and lack of understanding of the biology of mouse pox, particularly in inbred strains of mice.

To obtain data essential in developing strategies for prevention and control, a systematic study of the epizootiology of mousepox was initiated in April, 1980. It was necessary to develop a computer based system for the collection, storage, and processing of data. For this purpose, three separate but related data forms were devised for the collection of data on individual confirmed and presumptive cases, the population at risk within a room, and on conditions and environmental factors in the room in which an outbreak had occurred. The collection of data in this manner provided denominators as well as numerators for the calculation of incidence and prevalence, by time, place, and other variables such as genotype of mouse. These forms were initially utilized at NIH and then later made available to investigators at Washington University and St. Louis University Medical School in St. Louis, and the University of Chicago Medical School, where outbreaks had occurred or were in progress.

At NIH, the prevalence of diagnosed cases in three rooms from which adequate data was available (one room on campus, two off-campus at a contract facility) was respectively 3% of 939, 3% of 541 and 1% of 789 mice. The salient feature of the epizootic was slow and minimal spread of virus and consequent localization of cases. In one room at NIH, all but one of 28 cases were found on 1 of 5 racks in the room. Of 27 cases for which locations on the rack were known, all were limited to 11 cages, located on three adjacent shelves. The prevalence within the respective cages ranged from 20 to 100%. In one room at a contract facility 4 cases were detected in 3 cages located on 2 of 5 racks in the room. Each of the four predominant inbred strains (CBA, Balb/c, C57BL6 and C3H) present in one of the involved NIH rooms was found infected, with respective prevalences of 7%, 5%, 1% and 1%. Of the six predominant strains, D x B, Balb/c, C57BL/6,C57BL/10, C3H and DBA represented in one room at the contract facility, cases were found in the first two strains listed, with 4% and 3% prevalences respectively. In one NIH room, cases were limited to mice belonging to one of nine groups of investigators who used the room. There was no similar limitation of infected mice to particular investigators in the other rooms where this information was available. The source of the epizootic probably was the introduction of mice to one animal room at NIH from an infected mouse colony in an other state. Infected mice had been present in this room for approximately six months before the diagnosis was made. Circumstantial evidence suggested that the virus was spread by the movement of infected mice from this room to the contract facility and from there to the other NIH room. In spite of intensive surveillance efforts at NIH and several contract facilities (including the finding of HI-positive sera in some mice) there was not conclusive evidence that ectromelia virus had spread beyond the rooms referred to above. Data being collected at other institutions have not yet been analyzed; however, it is obvious from preliminary observations that the virus had been present for relatively long periods of time in certain inbred strains of mice before recognition. Most of the information in the literature on mousepox was obtained from studies done on outbred strains of mice (20 or more years ago before inbred strains came into wide use) exquisitely sensitive to the virus. It is now apparent that the epizootiology of mousepox differs among inbred strains.

The data from our studies, along with new experimental data,(see Annual Report # Z01 Al 00209-01 OSD) should help provide the basis for seriously considering a new strategy in the prevention and control of mouse pox. Previously, the strategy has been to destroy infected colonies as soon as possible, decontaminate the room and repopulate. This was reasonable, although costly, when only outbred strains of mice were being used and the disease was not endemic. However, it is not reasonable to destroy irreplaceable genetic stock. If it can be established through further experimentation and careful observation of epizootics that chronic or latent infections are not a factor in the natural history of ectromelia virus, and a specific and sensitive serologic assay for ectromelia infection can be developed (as seems likely), then it should be possible to eliminate the virus from a mouse colony by serologic monitoring and removal of cages housing serologic positive mice, rather than destroying the whole colony. Also this strategy can be supplemented with vaccination.

Future plans include the completion of studies on outbreaks at Washington University and Chicago University, and collaboration with others wishing to study other outbreaks. The establishment of a national mousepox surveillance program, through the Institute of Laboratory Animal Resources, National Research Council, National Academy of Science is in progress. It is hoped that this will provide information necessary to determine if ectromelia has become endemic in this country and allow for the development of procedures adequate to eradicate the virus.

Publications:

Wallace, G.D.: Mousepox threat. Science 211: 438, 1981.

Wallace, G.D.: Epizootiology of an outbreak of mousepox at the National Institutes of Health. Lab. Anim. Sci. In press.

LABORATORY OF BIOLOGY OF VIRUSES
1981 Annual Report
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Annual Report of the Laboratory of Biology of Viruses
National Institute of Allergy and Infectious Diseases, NIH
October 1, 1980 to September 30, 1981

The goal of the Laboratory of Biology of Viruses is to determine how viruses replicate. At the molecular level, this involves investigation of the structure of the virion and of its purified component parts, of mechanisms of replication and transcription and expression of the viral genome. Emphasis is also placed on cell-virus interactions including the process of cell transformation. We believe that information of this kind, although difficult to obtain, is fundamental to understanding disease processes and vital to achieving a rational basis for chemotherapy.

Papovaviruses

In Vitro Transcription of SV40 DNA by Purified RNA Polymerase II

RNA chains were found to be covalently linked to the 3' OH DNA terminus at the nicks. Appropriate treatments with exonuclease VII or S1 nuclease showed that RNA elongation was accompanied by the displacement of the non-coding DNA strand. DNAase I digestion of the DNA-RNA adduct showed that successive discrete termination events occurred at defined sites on SV40 DNA and that their relative efficiency was influenced by the salt concentration. Such discrete termination sites could play a role as "attenuators" or transitory pause sites for the RNA polymerase, and function as a regulatory control of the extent and/or the rate of transcription *in vivo*.

Covalently closed supercoiled or relaxed ciruclar SV40 DNAs were also used as templates. In both cases, initiation occurred at internal sites of the DNA, and there was no covalent attachment to the DNA template. Superhelical SV40 DNA was the most efficiently used template. Localization of the specific sites where initiation of RNA synthesis occurred *in vitro* showed that the conformation of the DNA drastically affects the selection of promoters by mammalian RNA polymerase II. Some of the major initiation sites were found to be located in close proximity to the promoters utilized *in vivo*. Our observations will be useful to determine the role and mechanism of action of the various cellular factors which have recently been shown to allow specific initiation of transcription to take place at natural promoter sites *in vitro*.

Stable Association of Viral Protein VP_1 with SV40 DNA

Previous transcription studies suggest that the association of viral proteins VP1 and/or VP2 with mature viral chromatin enhances transcriptional activity. The association of VP1 within the chromatin complex is mediated at least partially through a strong "interaction" with the viral DNA. Treatment of the virion chromatin complex with 0.25% sarkosyl dissociates VP2, VP3 and histones, leaving a stable VP1-DNA complex. Preliminary isoelectric focusing analysis of the VP1 associated with mature virion chromatin suggests that only two of the six virion VP1 species is present. It is plausible then, that the interaction of these specific VP1 species with virion chromatin regulates transcriptional activity. Sarkosyl treatment of SV40 chromatin should enable us to isolate these specific VP1 species with a high degree of purity and studies involving DNA binding, histone binding and post-translational modifications of VP1 in relation to transcription are now being carried out.

Site Specific Mutations that are Introduced in Regulatory Control Regions of the SV40 Genome.

We have introduced mutations at specific sites in the viral genome and then have monitored how these mutational events have altered the normal regulatory processes.

Two parallel experimental approaches have been used. In one case, a small plaque variant of SV40 has been isolated and cloned in a bacterial vector. Purified recombinant DNA was nicked at a unique SV40 *KpnI* site in the presence of ethidium bromide and the restriction enzyme. The nicked molecules were treated with exonuclease III to generate a population that had a single stranded region 100 nucleotides in length upstream or downstream with the nick forming one terminus. The DNA spanned by the single stranded gap does not include any information for a structural gene. The gapped molecules were treated with sodium bisulfite under conditions that lead to conversion of cytosine to thymine. The resultant DNA molecules were amplified in *E. coli* and the DNA was extracted from individual colonies. The SV40 portion was restricted from the vector and analyzed.

In the second procedure, the SV40 DNA was mutagenized at the *BglI* site, which is at or near the origin of replication and is also at or near the early promoter region. After mutagenesis, the DNA was cloned in a bacterial vector and individual recombinants were selected and analyzed. Out of 80 clones, only eight exhibit a loss of the *BglI* site. About eighteen clones show larger plaque morphology and six gave smaller plaques at 32°C compared to the wild type. About sixteen clones have been classed as lethal and these include four (out of eight) *Bgl*-resistant mutants.

Analysis of these mutants by sequencing, and by *in vitro* and *in vivo* transcription studies will define how regulatory signals are constructed in eucaryotic cells.

Accurate Initiation of RNA polymerase II on SV40 Viral Chromatin

In vitro RNA transcription systems have recently been developed which allow accurate initiation of RNA polymerase II on purified DNA templates. Since transcriptionally active eukaryotic genes are complexed with proteins as chromatin units *in vivo*, one aspect in understanding gene regulation is to determine what effect chromatin structure has on the expression of specific RNA synthesis.

We have previously demonstrated that mature SV40 virions contain a highly active transcription template which has properties similar to transcriptionally active eukaryotic genes. Following cleavage of chromatin DNA with endonculease Eco R1 to create a specific termination site, the incubation of SV40 chromatin with HeLa cell extracts results in the synthesis of three discrete RNA transcripts. Analyses of these three transcripts demonstrate that using SV40 chromatin as a template, one transcript originates at or near the in vivo early SV40 promoter site while the two other transcripts originate at or near in vivo late SV40 promoter sites. A comparison of RNA transcripts synthesized from naked SV40 DNA and SV40 chromatin indicates that specific transcription of the chromatin template is less affected when its DNA concentration is varied. In addition, fluctuation in specific promoter usage as a function of DNA concentration is not observed with the chromatin template. Further analysis of the 5' ends of RNA transcripts is required to determine if the chromatin template suppresses non-specific RNA transcription.

Parvoviruses

In Vivo Synthesis of Viral RNAs and Proteins

We have studied transcription and protein synthesis of the viral RNA in the KRV infected cell and uninfected cell and also in a cell free system. Utilizing DNA-RNA hybridization and the techniques of northern and southern blotting from electrophoresis gels to membrane papers, we have identified at least five species of viral RNA. The RNAs are transcribed from the infected viral DNA and have the approximate lengths of 4.6, 2.5, 1.7, 1.1 kilobases (Kb). These RNAs are probably functional messages since they were isolated from the polysomal fraction of the cell. The 4.6 Kb RNA would represent a transcript of 95 to 100% of the viral genome. The most abundant message about 3.1 Kb represents over 50% of the viral genome and probably codes in the reticulocyte transcribing system for the most abundant viral protein (MW 68,000) which is the main viral capsid protein. This abundant RNA, on the basis of R-loop data and hybridization to restriction enzyme fragments, has an origin of transcription about 3.8 to 4.2 map units from the 3' end of the single-stranded KRV genome. These viral specific RNAs may be synthesized individually from the viral genome or be the result of post transcriptional cleavage. The purified viral polysomal RNAs, when placed in a reticulocyte in vitro translation system, synthesize the known viral proteins in the virus capsid.

RNA Synthesis in Cell Free Systems

In an effort to study transcription and translation in more detail, we have developed a cell free system of isolated nuclei which synthesizes the five or six viral specific RNAs found in the in vivo infection. We have determined that the viral RNAs are polyadenylated on the 3' terminus and are transcribed solely from the viral strand of the virion, not from the complementary strand of the replicative intermediate. The

5' terminus of the RNA was shown by paper chromatography to be capped *in vitro* by the sequence $m^7G(5')ppp(5')A$. Incorporation of [β-^{32}P] ATP into the 5' cap sequence suggests that initiation of viral RNA synthesis may occur in the infected isolated nuclei. Since the major 4.6 kibopase RNA is synthesized abundantly in the nucleus, we hope to find out if this is a single transcriptional unit and the other viral RNAs are multiple spliced transcripts.

Adenoviruses

Since our initial description of the inverted terminal repetition (ITR) in adenovirus DNA, we have continued to investigate the possible biological and biochemical significance of this structure. We have now compared the genomes of several plaque isolates derived from the Ad 18 prototype strain to determine whether, for a given serotype, infectious genomes might contain ITRs of different length. Two isolates (P-1 and P-2) were found to have markedly different ITR lengths, and further analysis showed that (i) the approximately eight-fold longer P-2 ITR had been generated without any detectable deletion of adjacent internal sequences and (ii) these ITR genotypes remained essentially unchanged after repeated passage. In addition, comparisons of ITR base sequence from several adenovirus serotypes suggest that the junctional hexanucleotide (TGACGT) may be involved in determining ITR length. A continuing major objective of our studies will be to define the specific role of the ITR in viral replication.

Adenovirus-Associated-Viruses

The main objectives in studying defective human parvoviruses (AAV) are to define specific biochemical mechanisms used in synthesizing their DNA, RNA and proteins, to identify and characterize the helper virus-mediated step(s) required for their replication, to relate biochemical findings to normal cellular processes, and to determine conditions for selective interference of both AAV and Helper virus (adenoviruses, herpesviruses) infection.

Mechanism of AAV DNA Synthesis

AAV DNA replicative intermediates have been synthesized in soluble extracts prepared from infected cell nuclei, and conditions that yield *de novo* initiations of DNA synthesis, as well as elongation of molecules already initiated *in vivo*, have been defined. Analysis of the kinetics of DNA synthesis and of structural features of completed products support our initially reported model for the self-primed replication of AAV DNA. Further refinement of this system has been obtained by fractionation of extracts and utilization of exogenously added AAV DNA templates. Both virus and cell-specified requirements have been identified. It is likely that mechanisms involved in cell and AAV DNA synthesis possess important features in common. An ultimate goal is to determine conditions needed for repeated reinitiations of DNA synthesis *in vitro*.

Adenovirus Genes Required for Replication of AAV

We previously demonstrated that AAV multiplication is dependent upon the expression of one or more early adenovirus genes. Based on DNA transfection experiments, we have now mapped the locations of the required genes in the adenovirus genome (types 2 and 5). These genes map within three of the five known early regions (E1A, E2 and E4) and at the location of the VAI RNA gene. The requirement for this latter gene represents the first demonstration of its biological activity. E2 specifies only the first demonstration of its biological activity. E2 specifies only the 72,000 dalton DNA-binding protein which has been implicated in adenovirus DNA synthesis. Actual functions provided by the required adenovirus genes are being analyzed by further experiments. In most instances, it is likely that these genes play similar roles in the replication of the helper adenovirus. Clearly, AAV provides a useful marker for studying the individual roles of early, regulatory adenovirus genes. It is at this stage of the replication cycle that virus growth may be most vulnerable to selective interference.

Regulatory Activity of the Adenovirus DNA-Binding Protein

The adenovirus 72K DNA-binding protein synthesized during the early period of adenovirus infection is the most abundant of the viral regulatory proteins. It is multifunctional in that it is required for Ad (and AAV) DNA synthesis and for the coordinate expression of viral structural proteins (both Ad and AAV). We have extensively purified 72K protein from adenovirus infected and transformed cell lines and have studied its intracellular distributions by selective extraction and also by immunofluorescent staining. We have used these techniques to characterize the structural modifications of the 72K molecule as related to the efficient expression of Ad and AAV structural polypeptides *in vivo*.

Further studies are now in progress to utilize purified 72K protein for *in vitro* biosynthetic reactions. The major objective is to define specific interactions between 72K protein and other viral and host cell molecules that regulate viral infection.

Herpes Simplex Viruses Completely Help AAV Replication

In addition to adenoviruses, which are capable of completely helping AAV multiplication, only herpesviruses were known previously to provide any AAV-helper activity, but this activity was thought to be partial (i.e., AAV, DNA, RNA, and protein synthesis are induced but infectious particles are not assembled). We have now shown, however, that herpes simplex virus type 1 (HSV-1 and type 2 (HSV-2) are, in fact, complete AAV helpers, and that AAV type 2 (AAV-2) infectivity yields can approach those obtained when coinfections are carried out with a helper adenovirus. It should now be considered that, in addition to adenoviruses, herpes viruses may perpetuate AAV in nature and could contribute to the occurrence of widespread AAV infection in man. Furthermore, the known intracellular persistence of herpesviruses might conceivably promote persistence of AAV in certain human cells, a feature that could have pathogenic implications.

Poxviruses

I. Post-transcriptional modification of eukaryotic RNA. Members of this research group previously identified the 5' terminal cap structure $m^7G(5')pppN$, consisting of a 7-methylguanosine (m^7G) linked by a 5' to 5' triphosphate bridge to one or two consecutive 2'-O-methylated ribonucleosides (N^m) present in viral and cellular mRNAs. The mechanism of cap formation was determined by purifying and characterizing the responsible enzymes from vaccinia virus. Subsequently, by isolating enzymes from HeLa cells, we demonstrated that cap formation of eukaryotic and viral mRNAs occur by similar, although slightly different mechanisms. During the past year, we have extended this work by demonstrating a covalent HeLa cell capping enzyme - GMP intermediate and by separating and purifying HeLa cell cap I and cap II RNA (nucleoside-2'-) methyltransferases.

A. Identification of covalent GMP-capping enzyme intermediates. Previous studies from this laboratory indicated that capping enzyme purified from HeLa cells catalyzes the transfer of a GMP residue from GTP to a diphosphate terminus at the 5' end of RNA. Further studies now indicate that a stable covalent enzyme-GMP intermediate is formed. This intermediate can undergo the following two reactions:

$$\text{E-GMP} + \text{PPi} \rightleftharpoons \text{E} + \text{GTP}$$

$$\text{E-GMP} + \text{ppN-} \longrightarrow \text{E} + \text{G(5')pppN-}$$

in which ppN- is the diphosphate end of an RNA molecule. A phosphoamide linkage was demonstrated and a single tryptic GMP-peptide was isolated.

B. Purification and characterization of cap I and cap II RNA (nucleoside-2'-) methyltransferases. The existence of two separate RNA (nucleoside-2'-) methyltransferases involved in the modification of HeLa cell mRNA was established using assays that specifically measure the conversion of Cap 0 [$m^7G(5')pppN$-] to cap I [$m^7G(5')pppN^m$-] and cap I to cap II [$m^7G(5')pppN^mpN^m$-]. Cap II methyltransferase activity was found almost exclusively in cytoplasmic fractions while cap I methyltransferase activity was also found in the nucleus, its apparent biological site of action. The two enzymes were purified by ion exchange chromatography and their optimal reaction conditions determined. The finding that a capped terminus with at least two additional nucleotides is necessary for cap I methyltransferase activity indicates that initiation of transcription minimal chain extension and capping must precede 2'-O-methylation.

II. Organization and transcription of the vaccinia virus genome. Vaccinia virus, a member of the poxvirus family, provides a unique system for studying the synthesis and processing of mRNA from a DNA genome; all enzymes necessary for the synthesis of functional mRNA are contained within the infectious virus particle. The goal of this project is to define the roles of all enzymes and protein factors involved in the regulation of transcription. During the past year, virtually the entire genome of vaccinia virus was cloned in phage and plasmid vectors and a preliminary transcriptional/translational map was made. In addition, the left 20,000 bp of the genome was examined in more detail to better understand the organization of transcriptional units.

A. Arrangement of early and late mRNAs within the vaccinia virus genome. To complete a preliminary transcriptional/translational map of the entire vaccinia virus genome, the *HindIII* fragments N, M, K, F, E, O, I, G, L, J, H and D fragments were cloned in the plasmid pBR322. The recombinant DNAs were immobilized on nitrocellulose filters and used for hybridization to early and late mRNAs. The selected mRNAs were translated in the reticulocyte cell-free system and [^{35}S]methionine-labeled polypeptides were analyzed by polyacrylamide gel electrophoresis. Approximately 75 early and 40 late polypeptides were mapped. The major late polypeptides were clustered near the center of the genome whereas early mRNAs were distributed throughout.

B. Extension of the detailed transcriptional and translational map to 21 kilobase pairs. The second and third *EcoRI* fragments from the left end of the genome were cloned in phage λ. A variety of techniques including northern blotting, nuclease S1 mapping, *in vitro* cap labeling, cell-free translation of hybridization selected mRNAs, and hybridization arrest of translation were used to characterize and map mRNAs. A total of 11 early mRNAs encoding polypeptides of 6 to 60K and two late mRNAs encoding polypeptides of 22K and 40K were analyzed. The majority of early and late mRNAs were transcribed from the leftward reading strand, indicating that temporal regulation of expression does not occur by strand switching. Intervening sequences were not detected in any of the mRNAs suggesting that splicing does not occur during vaccinia virus mRNA synthesis.

C. Deletion of a 9,000 base pair segment of the vaccinia virus genome that encodes non-essential polypeptides. A naturally occuring deletion mutant of vaccinia virus was isolated. Hybridization selection and cell-free translation indicated that these variants lost the ability to synthesize at least eight early mRNAs mapping within the deleted region. Although the deleted DNA was not essential for replication of the WR strain of vaccinia virus under laboratory conditions of infection, it presumably has a defined role under other circumstances. This conclusion was based on the conservation of these sequences in other orthopoxviruses. The possibility that this region encoded polypeptides necessary for extra cellular viral transmission and virulence was suggested.

D. Distinctive nucleotide sequences adjacent to multiple initiation and termination sites of an early vaccinia virus gene. The 5'- and 3'-ends of an early mRNA synthesized *in vivo* and *in vitro* were shown to be heterogeneous by analysis of its capped oligonucleotides and the DNA segments that the RNA protects from single strand nuclease digestion. A remarkable 88% AT-rich 60 bp DNA sequence occurs immediately upstream of the major initiation site. Although sequences that bear some homology to Pribnow and Hogness boxes are present, additional recognition sequences found further upstream of prokaryotic and eukaryotic initiation sites are absent. The transcript terminates near or within a hexanucleotide CTATTC that is tandemly repeated four times. Sequences similar to those singaling transcriptional termination in prokaryotes or poly(A) addition in eukaryotes are not found suggesting that poxviruses have evolved unique recognition signals.

Construction of Vaccinia DNA Restriction Maps

The restriction enzymes BglI and SmaI cleave vaccinia DNA into five segments with masses of 48, 28, 24, 14, and 7 X 10^6 daltons. These segments can be recovered in 100 μg amounts by gradient centrifugation and they can be cut by other restriction enzymes, such as SalI, HindIII, XhoI and KpnI to produce groups of segments which can be ordered into physical maps. The smaller segments generated by a restriction digest of an individual Bgl segment can be mapped by several techniques. One system which I have developed uses digestion of a large segment such as Bgl-D by λ exonuclease which removes nucleotides from the 5' end of the DNA. This DNA is then digest with another enzyme such as HindIII. If the λ exonuclease digestion is extensive enough to reach an internal HindIII segment, it will remove one strand from one end of that segment so that it will not appear in the electrophoretic pattern of the HindIII digest. Sequential removal of internal segments allows the ordering of these segments. Often, this technique requires a rough location of a small (e.g. KpnI) segment on a segment produced by another enzyme such as HindIII, since intact vaccinia DNA is too large to analyze in this manner. λ exonuclease, unlike exonuclease III, does not attack at nicks appears to be processive. This method located small KpnI segments which allowed completion of the KpnI map. So far, HindIII, SalI, BglI, XhoI, SacI and KpnI physical maps have been determined for vaccinia virus DNA.

Honors and Awards

Dr. Norman P. Salzman continued to serve on the Editorial Board of the Journal of Virology, the Scientific Board of the Coordinating Council for Career Research, was a member of the NIAID Promotion and Tenure Committee, and was on the Editorial Advisory Board, Biochemistry. He serves as Professorial Lecturer, Georgetown University School of Medicine.

Dr. Moss continued to serve on the editorial boards of Journal of Virology, of Antibiotics and Chemotherapy, and of Intervirology, and as associate editor of Virology. In addition, he was appointed to the editorial board of the Journal of Biological Chemistry to start in 1982. Dr. Moss was appointed an American Society of Microbiology Foundation Lecturer and gave keynote addresses at the Michigan, Northern California and Washington local branches. He also served as co-chairman of the Poxvirus Workshop at the International Congress for Virology and was elected chairman of the 1983 Animal Cells and Virus Gordon Conference.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01AI 00123-15 LBV

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of viral and eukaryotic gene expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Bahige Baroudy	Visiting Fellow	LBV, NIAID
	Mary Haffey	Staff Fellow	LBV, NIAID
	Sundararajan Venkatesan	Expert Consultant	LBV, NIAID
	Jerry Weir	Guest Investigator	LBV, NIAID

COOPERATING UNITS (if any)

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Laboratory of Biology of Viruses

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.3	6.3	3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our primary objective is to determine the molecular events that regulate viral and eukaryotic gene expression. A better understanding of these processes may lead to effective antiviral chemotherapy. During the past year, we have continued studies on the enzymes that modify the 5' end of viral and cellular mRNA. The mechanism of capping was deduced by the demonstration of a HeLa cell capping enzyme - GMP covalent intermediate similar to that previously found for vaccinia virus capping enzyme. The isolation of two separate HeLa cell RNA (nucleoside-2'-)methyltransferases that catalyze the formation of cap I and cap II structures, respectively, extended our knowledge of RNA processing. Because vaccinia virus is an excellent system for studying gene expression, considerable effort was devoted to the mapping of viral RNA transcripts. Virtually the entire genome has now been cloned in phage or plasmid vectors and a preliminary map of more than 70 early and 40 late polypeptides was constructed. More detailed analysis of mRNAs encoded within the left 20 kilobase pairs of the genome indicated the absence of RNA splicing. DNA sequence analysis suggested that the vaccinia virus RNA polymerase recognizes unique signals for initiation and termination of transcription.

PHS-6040
(Rev. 10-76)

Major Findings

I. Post-transcriptional modification of eukaryotic RNA. Members of this research group previously identified the 5' terminal cap structure $m^7G(5')pppN$, consisting of a 7-methylguanosine (m^7G) linked by a 5' to 5' triphosphate bridge to one or two consecutive 2'-O-methylated ribonucleosides (N^m) present in viral and cellular mRNAs. The mechanism of cap formation was determined by purifying and characterizing the responsible enzymes from vaccinia virus. Subsequently, by isolating enzymes from HeLa cells, we demonstrated that cap formation of eukaryotic and viral mRNAs occur by similar, although slightly different mechanisms. During the past year, we have extended this work by demonstrating a covalent HeLa cell capping enzyme - GMP intermediate and by separating and purifying HeLa cell cap I and cap II RNA (nucleoside-2'-) methyltransferases.

A. Identification of covalent GMP-capping enzyme intermediates. Previous studies from this laboratory indicated that capping enzyme purified from HeLa cells catalyzes the transfer of a GMP residue from GTP to a diphosphate terminus at the 5' end of RNA. Further studies now indicate that a stable covalent enzyme-GMP intermediate is formed. This intermediate can undergo the following two reactions:

$$\text{E-GMP} + \text{PPi} \rightleftharpoons \text{E} + \text{GTP}$$

$$\text{E-GMP} + \text{ppN-} \longrightarrow \text{E} + \text{G(5')pppN-}$$

in which ppN- is the diphosphate end of an RNA molecule. A phosphoamide linkage was demonstrated and a single tryptic GMP-peptide was isolated.

B. Purification and characterization of cap I and cap II RNA (nucleoside-2'-) methyltransferases. The existence of two separate RNA (nucleoside-2'-) methyltransferases involved in the modification of HeLa cell mRNA was established using assays that specifically measure the conversion of Cap 0 [$m^7G(5')pppN$-] to cap I [$m^7G(5')pppN^m$-] and cap I to cap II [$m^7G(5')pppN^m pN^m$-]. Cap II methyltransferase activity was found almost exclusively in cytoplasmic fractions while cap I methyltransferase activity was also found in the nucleus, its apparent biological site of action. The two enzymes were purified by ion exchange chromatography and their optimal reaction conditions determined. The finding that a capped terminus with at least two additional nucleotides is necessary for cap I methyltransferase activity indicates that initiation of transcription minimal chain extension and capping must precede 2'-O-methylation.

II. Organization and transcription of the vaccinia virus genome. Vaccinia virus, a member of the poxvirus family, provides a unique system for studying the synthesis and processing of mRNA from a DNA genome; all enzymes necessary for the synthesis of functional mRNA are contained within the infectious virus particle. The goal of this project is to define the roles of all enzymes and protein factors involved in the regulation of transcription. During the past year, virtually the entire genome of vaccinia virus was cloned in phage and plasmid vectors and a preliminary transcriptional/translational map was made. In addition, the left 20,000 bp of the genome was examined in more detail to better understand the organization of transcriptional units.

A. Arrangement of early and late mRNAs within the vaccinia virus genome. To complete a preliminary transcriptional/translational map of the entire vaccinia virus genome, the HindIII fragments N, M, K, F, E, O, I, G, L, J, H and D fragments were cloned in the plasmid pBR322. The recombinant DNAs were immobilized on nitrocellulose filters and used for hybridization to early and late mRNAs. The selected mRNAs were translated in the reticulocyte cell-free system and [^{35}S]methionine-labeled polypeptides were analyzed by polyacrylamide gel electrophoresis. Approximately 75 early and 40 late polypeptides were mapped. The major late polypeptides were clustered near the center of the genome whereas early mRNAs were distributed throughout.

B. Extension of the detailed transcriptional and translational map to 21 kilobase pairs. The second and third EcoRI fragments from the left end of the genome were cloned in phage λ. A variety of techniques including northern blotting, nuclease S1 mapping, in vitro cap labeling, cell-free translation of hybridization selected mRNAs, and hybridization arrest of translation were used to characterize and map mRNAs. A total of 11 early mRNAs encoding polypeptides of 6 to 60K and two late mRNAs encoding polypeptides of 22K and 40K were analyzed. The majority of early and late mRNAs were transcribed from the leftward reading strand, indicating that temporal regulation of expression does not occur by strand switching. Intervening sequences were not detected in any of the mRNAs suggesting that splicing does not occur during vaccinia virus mRNA synthesis.

C. Deletion of a 9,000 base pair segment of the vaccinia virus genome that encodes non-essential polypeptides. A naturally occurring deletion mutant of vaccinia virus was isolated. Hybridization selection and cell-free translation indicated that these variants lost the ability to synthesize at least eight early mRNAs mapping within the deleted region. Although the deleted DNA was not essential for replication of the WR strain of vaccinia virus under laboratory conditions of infection, it presumably has a defined role under other circumstances. This conclusion was based on the conservation of these sequences in other orthopoxviruses. The possibility that this region encoded polypeptides necessary for extra cellular viral transmission and virulence was suggested.

D. Distinctive nucleotide sequences adjacent to multiple initiation and termination sites of an early vaccinia virus gene. The 5'- and 3'-ends of an early mRNA synthesized in vivo and in vitro were shown to be heterogeneous by analysis of its capped oligonucleotides and the DNA segments that the RNA protects from single strand nuclease digestion. A remarkable 88% AT-rich 60 bp DNA sequence occurs immediately upstream of the major initiation site. Although sequences that bear some homology to Pribnow and Hogness boxes are present, additional recognition sequences found further upstream of prokaryotic and eukaryotic initiation sites are absent. The transcript terminates near or within a hexanucleotide CTATTC that is tandemly repeated four times. Sequences similar to those signaling transcriptional termination in prokaryotes or poly(A) addition in eukaryotes are not found suggesting that poxviruses have evolved unique recognition signals.

Publications

Wittek, R. and Moss, B. Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell 21:277-284, 1980.

Wittek, R., Cooper, J. A., Barbosa, E., and Moss, B. Expression of the vaccinia virus genome: analysis and mapping of mRNAs encoded within the inverted terminal repetition. Cell 21:487-493, 1980.

Keith, J. M., Gershowitz, A., and Moss, B. Dinucleotide sequences at the 5'-ends of vaccinia virus mRNAs synthesized in vitro. J. Virol. 36:601-605, 1980.

Cooper, J. A., Wittek, R., and Moss, B. Hybridization selection and cell-free translation of mRNAs encoded within the inverted terminal repetition of the vaccinia virus genome. J. Virol. 37:284-294, 1981.

Venkatesan, S., and Moss, B. In vitro transcription of the inverted terminal repetition of the vaccinia virus genome: correspondence of initiation and cap sites. J. Virol. 37:738-747, 1981.

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Wittek, R., Cooper, J. A., and Moss, B. Transcriptional and translational mapping of a 6.6 kilobase pair DNA fragment containing the junction of the terminal repetition and unique sequence at the left end of the vaccinia virus genome. J. Virol., in press.

Cooper, J. A., Wittek, R., and Moss, B. Extension of the transcriptional and translational map of the left end of the vaccinia virus genome to 21 kilobase pairs. J. Virol., in press.

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Langberg, S. R., and Moss, B. Posttranscriptional modifications of mRNA. Purification and characterization of cap I and cap II RNA (nucleoside-2'-)-methyltransferases from HeLa cells. J. Biol. Chem., in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00124-12 LBV

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Replication of the Parvovirus, KRV

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Lois A. Salzman	Research Biochemist	LBV, NIAID
Other:	Dr. Rita Mitra	Visiting Fellow	LBV, NIAID
	Ms. Phyllis Fabisch	Microbiologist	LBV, NIAID

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Laboratory of Biology of Viruses

SECTION
Molecular Structure Section

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NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	2	1.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are following the synthesis of the parvovirus, KRV, as a tool to understand both cellular and viral mRNA synthesis. Viral specific RNAs are synthesized in the nucleus, processed, modified and transported to the cytoplasm, presumably by the same mechanism as cellular mRNA biosynthesis. However, viral RNA synthesis involves fewer species of synthesized RNA than total cellular RNA. Hence, it is a simpler system for study than the complex eukaryotic cellular RNA. The viral RNAs can be identified as viral specific by hybridization, gel electrophoresis, restriction enzyme analysis and by Northern and Southern blotting techniques.

We have developed a cell free system in isolated nuclei which can synthesize viral specific RNA. We have also isolated the viral RNAs from infected cells and used the viral RNA to synthesize in vitro viral proteins. Further study should yield more detailed information on the requirements and conditions needed for RNA and protein synthesis in the infected and normal eukaryotic cell.

Project Description

The study of eukaryotic RNA synthesis is very complex because of the number of RNAs and the involved method of their synthesis in the animal cell. In an attempt to study RNA synthesis using a more simplified system, many workers have turned to the study of viral specific RNAs and proteins in the eukaryotic cell. Viral-specific RNAs are synthesized in the nucleus, processed, modified and transported to the cytoplasm presumably by the same mechanism as cellular mRNA biosynthesis. Thus, using viral infected cells and cell free systems, we can learn more about the requirements and control mechanisms used in synthesis of RNA and proteins. Learning about the process may aid in finding ways to control treatment and prevention of viral infection. We also learn more about the cellular mechanisms of RNA synthesis and can test for these mechanisms and pathways in normal and abnormal cells.

Our approach in the laboratory has been to use as a model system the parvovirus, KRV. The parvoviruses are the smallest eukaryotic viruses containing a single strand of DNA about 5,000 base in length and two or three proteins. The virus can only code for about three to six viral proteins, yet it infects the cell, turns the cell into a virus producing machine, and destroys the cell within 20 to 22 hours after infection.

We have studied transcription and protein synthesis of the viral RNA in the KRV infected cell and uninfected cell and also in a cell free system. Utilizing DNA-RNA hybridization and the techniques of northern and southern blotting from electrophoresis gels to membrane papers, we have identified at least five species of viral RNA. The RNAs are transcribed from the infected viral DNA and have the approximate lengths of 4.6, 2.5, 1.7, 1.1 kilobases (Kb). These RNAs are probably functional messages since they were isolated from the polysomal fraction of the cell. The 4.6 Kb RNA would represent a transcript of 95 to 100% of the viral genome. The most abundant message about 3.1 Kb represents over 50% of the viral genome and probably codes in the reticulocyte transcribing system for the most abundant viral protein (MW 68,000) which is the main viral capsid protein. This abundant RNA, on the basis of R-loop data and hybridization to restriction enzyme fragments, has an origin of transcription about 3.8 to 4.2 map units from the 3' end of the single-stranded KRV genome. These viral specific RNAs may be synthesized individually from the viral genome or be the result of post transcriptional cleavage. The purified viral polysomal RNAs, when placed in a reticulocyte *in vitro* translation system, synthesize the known viral proteins in the virus capsid. Further studies will reveal which RNA is responsible for the synthesis of each capsid protein. Using the S1 nuclease system, we hope to determine if one or all the viral RNAs are spliced and if they share common or individual leader sequences.

In an effort to study transcription and translation in more detail, we have developed a cell free system of isolated nuclei which synthesizes the five or six viral specific RNAs found in the *in vivo* infection.

We have determined that the viral RNAs are polyadenylated on the 3' terminus and are transcribed solely from the viral strand of the virion, not from the complementary strand of the replicative intermediate. The 5' terminus of the RNA was shown by paper chromatography to be capped *in vitro* by the sequence $m^7G(5')ppp(5')A$. Incorporation of [β-^{32}P] ATP into the 5' cap sequence suggests that initiation of viral RNA synthesis may occur in the infected isolated nuclei. Since the major 4.6 kibopase RNA is synthesized abundantly in the nucleus, we hope to find out if this is a single transcriptional unit and the other viral RNAs are multiple spliced transcripts. This is a good system for studying the mechanisms used in synthesis of RNA and we hope that it will lead to a better understanding of the complex eukaryotic cellular RNA synthesis.

Publications

1. Salzman, L. A., Fabisch, P., Mitra, R., and Wali, T. Parvovirus mRNA synthesis in isolated nuclei. J. Virol., in press.

2. Mitra, R., Wali, T., Valdez, V., Fabisch, P., and Salzman, L. A. In vitro synthesis and characterization of mRNA by the parvovirus, KRV. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00125-12 LBV

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Viral DNA Replication, Transcription, and Integration

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Norman P. Salzman	Chief	LBV, NIAID
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	Gokul Das	Visiting Associate	LBV, NIAID
	Christian Lavialle	Visiting Fellow	LBV, NIAID
	Michael Vodkin	Staff Fellow	LBV, NIAID
	Qian Xu	Guest Worker	LBV, NIAID

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TOTAL MANYEARS: 8	PROFESSIONAL: 6	OTHER: 2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

SV40 transcription has been examined after introduction of point mutations in regulatory control regions. These mutants show altered growth properties and differential utilization of promoters. SV40 chromatin complexes are transcribed efficiently and with greater specificity in vitro then is purified SV40 DNA. When purified SV40 is transcribed by purified calf thymus RNA polymerase II there is specificity in initiation and in RNA termination.

PHS-6040
(Rev. 2-81)

Project Description

In Vitro Transcription of SV40 DNA by Purified RNA Polymerase II

The transcriptional properties of highly purified calf thymus RNA polymerase II were investigated using SV40 DNA in various conformational states as a template. When nicked circular or linear SV40 DNAs were transcribed a major fraction of the RNA appeared to be covalently linked to the DNA, as a consequence of the priming of RNA synthesis at the nicks or ends of the DNA. In order to study the mechanism of this reaction in more details, we used as a template, SV40 DNA that had been nicked at a single specific site by incubation with the restriction endonucleases Bgl I or Hpa II in the presence of ethidium bromide. RNA chains were found to be covalently linked to the 3' OH DNA terminus at the nicks. Appropriate treatments with exonuclease VII or S1 nuclease showed that RNA elongation was accompanied by the displacement of the non-coding DNA strand. DNAase I digestion of the DNA-RNA adduct allowed us to demonstrate that successive discrete termination events occurred at defined sites on SV40 DNA and that their relative efficiency was influenced by the salt concentration. Such discrete termination sites could play a role as "attenuators" or transitory pause sites for the RNA polymerase, and function as a regulatory control of the extent and/or the rate of transcription in vivo.

Covalently closed supercoiled or relaxed ciruclar SV40 DNAs were also used as templates. In both cases, initiation occurred at internal sites of the DNA, generating transcripts unaffected by DNAase treatment. Superhelical SV40 DNA was the most efficiently used template. When the $(NH_4)SO_4$ concentration was increased to 125 mM, following initiation of transcription the rate of RNA chain elongation was maximal, leading to the synthesis of RNA molecules greater than full-length SV40 DNA. Further increasing the salt concentration caused termination to occur at specific sites on the DNA, as demonstrated by the appearance of a discrete RNA band pattern. Localization of the specific sites where initiation of RNA synthesis occurred in vitro showed that the conformation of the DNA drastically affects the selection of promoters by mammalian RNA polymerase II. Some of the major initiation sites were found to be located in close proximity to the promoters utilized in vivo. Our observations will be useful to determine the role and mechanism of action of the various cellular factors which have recently been shown to allow specific initiation of transcription to take place at natural promoter sites in vitro.

Site Specific Mutations that are Introduced in Regulatory Control Regions of the SV40 Genome.

During the process of viral replication, viral genes are activated at specific times during the infectious cycle. The present studies were carried out to understand the mechanisms of gene regulation. We have introduced mutations at specific sites in the viral genome and then have monitored how these mutational events have altered the normal regulatory processes.

Two parallel experimental approaches have been used. In one case, a small plaque variant of SV40 has been isolated and cloned in a bacterial vector. Purified recombinant DNA was nicked at a unique SV40 *KpnI* site in the presence of ethidium bromide and the restriction enzyme. The nicked molecules were treated with exonuclease III to generate a population that had a single stranded region 100 nucleotides in length upstream or downstream with the nick forming one terminus. The DNA spanned by the single stranded gap does not include any information for a structural gene. The gapped molecules were treated with sodium bisulfite under conditions that lead to conversion of cytosine to thymine. The resultant DNA molecules were amplified in *E. coli* and the DNA was extracted from individual colonies. The SV40 portion was restricted from the vector and analyzed. Some isolated DNAs no longer contained a *KpnI* site, suggesting that mutagenesis had occurred at the enzymatic recognition sequence. Some DNAs produced no plaques (lethal) or plaques with altered morphology, suggesting that the infectious cycle was perturbed.

In the second procedure, the SV40 DNA was mutagenized at the *BglI* site, which is at or near the origin of replication and is also at or near the early promoter region. After mutagenesis, the DNA was cloned in a bacterial vector and individual recombinants were selected and analyzed. Out of 80 clones, only eight exhibit a loss of the *BglI* site. About eighteen clones show larger plaque morphology and six gave smaller plaques at 32°C compared to the wild type. About sixteen clones have been classed as lethal and these include four (out of eight) *Bgl*-resistant mutants.

Analysis of these mutants by sequencing, and by *in vitro* and *in vivo* transcription studies will define how regulatory signals are constructed in eucaryotic cells.

Stable Association of Viral Protein VP_1 with SV40 DNA

Previous transcription studies suggest that the association of viral proteins VP1 and/or VP2 with mature viral chromatin enhances transcriptional activity. The association of VP1 within the chromatin complex is mediated at least partially through a strong "interaction" with the viral DNA. Treatment of the virion chromatin complex with 0.25% sarkosyl dissociates VP2, VP3 and histones, leaving a stable VP1-DNA complex. The VP1-DNA complex has a sedimenatation value of 30S and a density of 1.460 g/cc. The calculated molecular weight of the complex is 7.9×10^6 daltons with an average of 100 VP1 proteins per molecule of SV40 DNA.

Preliminary isoelectric focusing analysis of the VP1 associated with mature virion chromatin suggests that only two of the six virion VP1 species is present. It is plausible then, that the interaction of these specific VP1 species with virion chromatin regulates transcriptional activity. Sarkosyl treatment of SV40 chromatin should enable us to isolate these specific VP1 species with a high degree of purity and studies involving DNA binding, histone binding and post-translational modifications of VP1 in relation to transcription are now being carried out.

Accurate Initiation of RNA polymerase II on SV40 Viral Chromatin

In vitro RNA transcription systems have recently been developed which allow accurate initiation of RNA polymerase II on purified DNA templates. Combined with the analysis of RNA synthesized in vivo, these in vitro systems provide important information on the mechanisms involved in the regulation of RNA transcription in eukaryotic cells. Since transcriptionally active eukaryotic genes are complexed with proteins as chromatin units in vivo, one aspect in understanding gene regulation is to determine what effect chromatin structure has on the expression of specific RNA synthesis. SV40 chromatin provides a model template for such studies since the structure and mode of transcription closely resembles cellular chromosomes.

We have previously demonstrated that mature SV40 virions contain a highly active transcription template which has properties similar to transcriptionally active eukaryotic genes. Following cleavage of chromatin DNA with endonculease Eco Rl to create a specific termination site, the incubation of SV40 chromatin with HeLa cell extracts results in the synthesis of three discrete RNA transcripts approximately 3450(A), 1610(B) and 1485(C) nucleotides in length. Endonuclease restriction of the chromatin DNA with Eco Rl and Bam Hl decreases the length of transcript A by 750 nucleotides, while the length of transcripts B and C are unchanged. Endonuclease restriction of chromatin DNA with Eco Rl and Hae II leaves transcript A intact, but decreases the length of transcripts B and C by 950 nucleotides. Following hybridization of RNA transcripts to "early/late" SV40 DNA southern blots, the RNA was eluted and analyzed by glyoxal gel electrophoresis. Transcript A hybridized exclusively to the coding strand for early SV40 RNA while transcripts B and C hybridized to the DNA strand coding for late SV40 RNA. These analyses demonstrate that using SV40 chromatin as a template, transcript A originates at or near the in vivo early SV40 promoter site while transcripts B and C originate at or near in vivo late SV40 promoter sites. A comparison of RNA transcripts synthesized from naked SV40 DNA and SV40 chromatin indicates that specific transcription of the chromatin template is less affected when its DNA concentration is varied. In addition, fluctuation in specific promoter usage as a function of DNA concentration is not observed with the chromatin template. Further analysis of the 5' ends of RNA transcripts is required to determine if the chromatin template suppresses non-specific RNA transcription.

Structure of Intracellular Nucleoprotein Complexes

We have used recombinant clones which contain specific regions of the SV40 genome to probe the structure of intracellular SV40 nucleoprotein complexes. These complexes fall into three categories, those involved in replication; those involved in transcription; and intermediates in the assembly of virions. These complexes have been extracted at different times during the infectious cycle and nucleosomes have been formed by partial micrococcal nuclease digestion. We are examining if there is phasing of the nucleosomes which changes at different times during the infectious cycle. This may be an important determinant of promoter selection.

Publications

Lavialle, C., Reuveni, Y., Thoren, M., and Salzman, N. P. Molecular interaction between Simian Virus 40 and *Escherichia coli* RNA polymerase: Mapping of the initiation sites on supercoiled and linear DNA. Jrl. Biol. Chem., in press.

Brady, J., Lavialle, C., Radonovich, M., and Salzman, N. P. Stable association of viral protein VP1 with Simian Virus 40 DNA. J. Virol. 39, 1981.

Brady, J. Radonovich, M., Lavialle, C., and Salzman, N. P. Simian Virus 40 maturation: Chromatin modifications increase the accessibility of viral DNA to nuclease and RNA polymerase. J. Virol. 39, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00126-08 LBV

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Restriction ENzyme Analysis of Vaccinia Virus DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Frank DeFilippes, Research Physicist LBV NIAID

COOPERATING UNITS (if any)

Dr. Daniel Moynet, LSD, NIAID

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of the project is to study the organization of genes in large DNA molecules obtained from animal viruses. The production and ordering of unique segments of vaccinia virus DNA provide a physical map of the genome. Physical maps have been determined for several different restriction enzymes including SalI, HindIII, XhoI, KpnI, SmaI, and BglI. The last two enzymes are very useful since they cleave the DNA into five easily separated segments from which other restriction enzyme maps can be determined.

Viral DNA segments are being cloned in plasmids with the aim of doing sequence determination on the region containing the origin of replication and any possible signal elements in the viral genome.

DNA from phage N3 which exists as a prophage in Hemophilus influenzae was characterized. This aggregation of this DNA is caused by single strand tails at the termini. DNA from phage 42, which can lysogenize streptococci, was also studied and shown to be circularly permuted. A new method was developed for examining the extent of this circular permutation.

Project Description

The restriction enzymes *Bgl*I and *Sma*I cleave vaccinia DNA into five segments with masses of 48, 28, 24, 14, and 7 X 10^6 daltons. These segments can be recovered in 100 μg amounts by gradient centrifugation and they can be cut by other restriction enzymes, such as *Sal*I, *Hind*III, *Xho*I and *Kpn*I to produce groups of segments which can be ordered into physical maps. The smaller segments generated by a restriction digest of an individual Bgl segment can be mapped by several techniques. One system which I have developed uses digestion of a large segment such as *Bgl*-D by λ exonuclease which removes nucleotides from the 5' end of the DNA. This DNA is then digest with another enzyme such as *Hind*III. If the λ exonuclease digestion is extensive enough to reach an internal *Hind*III segment, it will remove one strand from one end of that segment so that it will not appear in the electrophoretic pattern of the *Hind*III digest. Sequential removal of internal segments allows the ordering of these segments. Often, this technique requires a rough location of a small (e.g. *Kpn*I) segment on a segment produced by another enzyme such as *Hind*III, since intact vaccinia DNA is too large to analyze in this manner. λ exonuclease, unlike exonuclease III, does not attack at nicks and appears to be processive. This method located small *Kpn*I segments which allowed completion of the *Kpn*I map. So far, *Hind*III, *Sal*I, *Bgl*I, *Xho*I, *Sac*I and *Kpn*I physical maps have been determined for vaccinia virus DNA.

λ exonuclease can also be used to reduce segments to smaller sizes since the protruding 3' strand can be trimmed by BAL 31 nuclease digestion. *Hind*III and *Sal*I segments are being cloned in plasmids pBR325, 327 and 328 in order to produce larger quantitites of these DNAs for nucleotide sequence analysis.

The DNA from phage N3 which can exist as a prophage in *Hemophilus influezae* strain *Rd* has been characterized. The phage is induced by mitomycin and purified by CsCl binding. THe DNA isolated from the phage is double stranded with a mass of 26.8 X 10^7 daltons. A 34% G-C content is shown by both equilibrium centrifugation and melting curves. The DNA has sticky ends which cause aggregation in buffers of moderate ionic strength. Heating the DNA for 10 minutes at 68°C in 10 mM Tris-Cl pH 8.3 will usually remove the association of the sticky ends. With some preparations of DNA, however, up to 20% of the DNA was not reduced to linear monomers by heating. S1 nuclease treatment at 48°C did remove all effects of the sticky ends, proving that single stranded DNA was responsible for molecular aggregation. Analyses of restriction enzyme digests were used to form physical maps of N3 DNA. *Eco*RI, *Xho*I, *Xba*I, *Kpn*I and *Sac*I maps have been determined.

The DNA from phage42, which can lysogenize or lyse group H streptococci, has also been characterized. This DNA is double stranded and has a mass of 24.1 X 10^6 daltons. The DNA has a terminal repetition and is circularly permuted. We developed a method for examining the circular permutation of this DNA using exonuclease III digestion followed by restriction enzyme cleavage. A non-permuted DNA from adenovirus 2 was used to show that exonuclease III digestion removed the terminal segments as shown by electrophoretic patterns of the restriction enzyme cleaved DNA. When this same procedure was used for 42 DNA, no particular segment disappeared indicating that these molecules did not contain a population with a definite nucleotide sequence at each terminus. The initial indication is that any particular sequence of the DNA is as likely as any other to appear at the termini. We believe this method for examining permuted molecules will be very useful to those people who do not have electron microscopes and that it will also extend the information of those groups who can examine permuted DNA with the classic EM procedure.

Publications

Filipska, E., Moynet, D., and DeFilippes, F. Characterization of N3 DNA. J. Virology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00127-14 LBV

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Structure and Function of Genetic and Protein Components of Defective Parvoviruses and their Helpers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
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Molecular Structure Section

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7	5	2

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Main objectives in studying these defective human parvoviruses (AAV) are to (i) define specific biochemical mechanisms used in synthesizing their DNA, RNA and proteins, (ii) identify and characterize the helper virus-mediated step(s) required for their replication and (iii) relate biochemical findings to normal cellular processes as well as to potentials for selective interference with both AAV and Helper virus (adenoviruses, herpesviruses) infection. Among the methods used are nucleic acid hybridizations, analytical and preparative sucrose and CsCl sedimentations, molecular cleavage with restriction nucleases, gel electrophoresis, electron microscopy, DNA transfection and DNA cloning.

Project Description

(1) We previously proposed (Straus, et al., 1976, Proc. Natl. Sci. U.S.A. 73:742) that a 3'-terminal segment of AAV DNA serves as the primer for AAV DNA synthesis. The resulting unit length duplex hairpin could then be converted to a full length, linear duplex molecule according to a mechanism proposed by Cavalier-Smith (1974, Nature 250, 467). This latter structure presumably serves as the template for displacement synthesis of plus or minus progeny strands. We have now demonstrated that these replicating intermediates can be synthesized in soluble extracts prepared from infected cell nuclei. Elongation of molecules already initiated in vivo as well as de novo initiation of synthesis on exogenously added DNA templates has been shown to occur. Extracts can be fractionated into sedimented and supernatant components which are both required for viral DNA synthesis. The supernatant component from uninfected cells can replace supernatant components derived from Ad- or Ad/AAV-infected cells. This indicates that all necessary virus-specific factors are present in the sedimented component. Both virus and cell-specified factors have been identified and are currently in the process of detailed characterization. Among our goals are (i) to gain insight into mechanisms involved in regulation of eukaryotic DNA synthesis and (ii) to determine conditions needed for the repetitive synthesis of DNA molecules in vitro.

(2) AAV multiplication is dependent upon the expression of one or more early adenovirus genes (Straus et al., 1976, J. Virol. 17:140; Richardson et al., 1980, Proc. Natl. Acad. Sci. U.S.A. 77:931). In our current studies, DNA transfection was used to map the locations of adenovirus type 2 or type 5 genes for replication of AAV. Three restriction endonuclease fragments of adenovirus DNA were required for AAV replication in 293-31 cells (an Ad 5-transformed human embryonic kidney cell line). These fragments map from 28.5-29.4, 59.5-75.9 and 89.7-100 map units on the adenovirus genome and correspond to the VAI RNA gene, the 72,000 dalton DNA-binding protein gene (early region 2) and early region 4, respectively. The 293-31 cell line expresses early region 1A and 1B proteins but alone was incapable of supporting AAV DNA or protein synthesis. AAV coinfections with host range mutants hr 1 and hr 7 indicate that AAV replication requires the 1A region, whereas the 1B region is probably not needed. Although it is not clear whether one or more early adenovirus gene products directly mediate AAV synthesis, these studies demonstrate that transfected DNA fragments which contain adenovirus genes from three early regions are capable of expressing functional products. Furthermore, utilizing the AAV genome as an indicator, our results reveal for the first time that the VAI RNA gene does possess a biosynthetic function. We plan to define the nature of specific biochemical interactions that are involved, since these early viral gene products carry out vital regulatory functions that serve as logical targets for selectively interfering with the process of viral infection.

(3) The adenovirus 72K DNA-binding protein synthesized during the early period of adenovirus infection is the most abundant of the viral regulatory proteins. It is multifunctional in that it is required for Ad (and AAV) DNA

synthesis and for the coordinate expression of viral structural proteins (both Ad and AAV). We have extensively purified 72K protein from adenovirus infected and transformed cell lines and have studied its intracellular distributions by selective extraction and also by immunofluorescent staining. We have used these techniques to characterize the structural modifications of the 72K molecule as related to the efficient expression of Ad and AAV structural polypeptides *in vivo*.

Further studies are now in progress to utilize purified 72K protein for *in vitro* biosynthetic reactions. The major objective is to define the specific interactions between 72K protein and other viral and host cell molecules that regulate viral infection.

(4) In addition to adenoviruses, which are capable of completely helping AAV multiplication, only herpesviruses were known previously to provide any AAV-helper activity, but this activity was thought to be partial (i.e., AAV, DNA, RNA, and protein synthesis are induced but infectious particles are not assembled). We have now shown, however, that herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are, in fact, complete AAV helpers, and that AAV type 2 (AAV 2) infectivity yields can approach those obtained when coinfections are carried out with a helper adenovirus.

To help locate and identify the function(s) of herpes simplex virus genes that are required for AAV multiplication, a number of HSV-1 and HSV-2 temperature-sensitive mutants are being screened for their ability to support AAV growth. These studies presently suggest that AAV multiplication depends on a product(s) specified by the herpesvirus DNA polymerase gene. It should now be considered that, in addition to adenoviruses, herpesviruses may perpetuate AAV in nature and could contribute to the occurrence of widespread AAV infection in man. Furthermore, the known intracellular persistence of herpesviruses might conceivably promote persistence of AAV in certain human cells, a feature that could have pathogenic implications.

PUBLICATIONS

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Buller, R. M. L., Janik, J. E., Sebring, E. D., and Rose, J. A. Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. J. Virology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00128-14 LBV

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Properties of Adenovirus DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Claude F. Garon	Research Microbiologist	LBV, NIAID
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1	0.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of these studies has been the application of physical and biochemical techniques to map and to define the structure of specific genetic regions in the genomes of both oncogenic and non-oncogenic human adenoviruses, with the ultimate goals of (1) relating these sequences to those biochemical activities which permit these viruses to influence or gain control of cellular functions (e.g., cell lysis and transformation) and (2) of defining basic mechanisms for regulation of genetic expression (e.g., initiation of DNA synthesis). A number of unique features have been described which may have implications in viral replication or carcinogenesis or both. Among the methods used are gradient sedimentation, molecular cleavage with restriction nucleases, gel electrophoresis, base sequence analysis, DNA cloning and electron microscopy.

Project Description

(1) We have continued an analysis of certain structural features of adenovirus DNA. A comparison of DNA from plaque isolates (P-1 and P-2) derived from the prototype strain of a highly oncogenic serotype (type 18) revealed P-2 DNA molecules are about 4% longer than P-1 molecules, and that the observed length difference is due to a longer inverted terminal repetition (ITR) in P-2 DNA. For both P-1 and P-2, this genotypic feature remained unchanged after five additional virus passages. These data strongly suggest that infectious adenovirus genomes may carry ITRs of different length. Based on direct nucleotide sequence analysis, the P-1 ITR is 165 bases long and possesses several short sequences in common with the ITRs of Ad2 and Ad 7 and extensive homology with the ITR of Ad 12. In all instances, the same hexanucleotide (TGACGT) occurs at or within two bases of the end of the ITR, suggesting that it might play a role in determining ITR length. Base sequence analysis studies are currently under way with P-2 DNA in an attempt to confirm the suggested role of this junctional hexanucleotide.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00201-02 LBV

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)
Structural Characterization of Integrated Viral Genomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	Nancy G. Schmidt	Biologist	LBV, NIAID
	Malcolm A. Martin		DNA, NIAID
	Takis S. Papas		NCI
	Charles J. Sherr		NCI

COOPERATING UNITS (if any)

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2.5	1.5	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several classes of viruses form stable associations with their hosts by the integration of one or more copies of the viral genome into host cell DNA. Retroviruses provide a unique and important system for the study of this process since exogenously acquired genomes are integrated at a specific site in the viral genome, but at a large number of sites in cellular DNA. Molecular clones of several newly integrated retroviral genomes were produced in either plasmid or bacteriophage cloning vehicles using approved recombinant DNA techniques and were characterized using electron microscope heteroduplex and R-looping methods. The major objective of these studies has been the application of physical and biochemical techniques to assess the influence of flanking cellular sequences on subsequent viral function and to define in molecular terms those events which take place during integrative recombination.

PHS-6040
(Rev. 10-76)

It has been shown in a number of retroviral systems that exogenously acquired genomes are integrated at a specific site in the viral genome, but at a large number of sites in cellular DNA. In order to investigate more closely this association between host cell and viral sequences, molecular clones of several newly integrated retroviral genomes were produced in either plasmid or bacteriophage cloning vehicles using approved recombinant DNA techniques and were studied using electron microscope heteroduplex methods. Electron microscope heteroduplex analysis is a sensitive method for detecting and mapping regions of sequence homology and non-homology between related nucleic acid species.

Avian myelocytomatosis virus (MC29), a defective acute leukemia virus, has a broad oncogenic spectrum *in vivo* and transforms fibroblasts and hematopoietic target cells *in vitro*. We have used recombinant DNA technology to isolate and to characterize the sequences that are essential in the transformation process. Integrated MC29 proviral DNA was isolated from a library of recombinant phage containing DNA from the MC29-transformed non-producer quail cell line Q5. The cloned DNA was analyzed by Southern blotting of restriction endonuclease digests and by electron microscopic visualization of R loops formed between the cloned DNA and MC29 or helper virus RNA. It was found that the 9.2-kilobase cloned DNA insert contains approximately 4 kilobases of viral sequences and 5.2 kilobases of quail cellular sequences. The viral sequences contain all of the MC29-specific sequences and 5' helper-related sequences as well as part of the envelope region. The size of the cloned *EcoRI* fragment is the same as that of the major band in *EcoRI*-cleaved Q5 DNA that hybridizes to viral sequences. Transfection of the cloned DNA into NIH 3T3 cells revealed that the MC29-specific sequences are functional in that they induce foci of transformed cells with high efficiency.

The integrated DNA provirus of the Gardner-Arnstein (GA) strain of feline sarcoma virus (FeSV) has been molecularly cloned in a bacteriophage ω vector. The cloned DNA fragment is 14.4 kilobase pairs (kb) in length and contains a 6.7 kb provirus flanked by cellular sequences derived from nonproductively transformed mink cells. Transfection of mouse NIH/3T3 cells with the cloned DNA fragment induced foci of transformation at efficiencies of 10-20,000 focus-forming units per picomole of sarcoma virus DNA. The GA-FeSV provirus was compared to that of Snyder-Theilen (ST)-FeSV, a second strain which contains homologous, transformation-specific sequences (v-fes). Both viruses have the general structure *5'-gag-fes-env-c* *region*-3', where only portions of the feline leukemia virus (FeLV) *gag* and *env* genes are represented. Restriction endonuclease and heteroduplex analyses indicated that GA-FeSV contains 1.7 kb of FeLV-derived genetic information not present in ST-FeSV. Of these, at least 400-500 base pairs located at the 5' end of *v-fes* encode a portion of the GA-FeSV polyprotein; the remaining 1.2 kb are derived from the *env* gene and do not appear to encode any detectable product related to the FeLV envelope glycoprotein. Thus, the major differences between the two isolates reside in sequences derived from FeLV. By contrast, the close homology of the v-*fes* sequences shows that GA- and ST-FeSV were formed by recombination of FeLV with very similar, and possibly identical, portions of a cat cellular gene (c-*fes*).

We propose to exploit the potential of recombinant DNA technology for the detection and amplification of rare DNA sequences such as a single, integrated viral genome in order to 1) learn more about the specificity of various integration events, 2) assess the influence of flanking cellular sequences on the subsequent functioning of the viral genome and 3) to define in molecular terms those events that take place during integrative recombination.

Publications

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Lautenberger, J. A., Schulz, R. A., Garon, C. F., Tsichlis, P. N., and Papas, T. S. 1981. Molecular cloning of avian myelocytomatosis virus (MC29) transforming sequences. Proc. Natl. Acad. Sci. USA 78:1518-1522.

Fedele, L. A., Even, J., Garon, C. F., Donner, L., and Sherr, C. J. 1981. Characterization of recombinant bacteriophages containing the integrated, transforming provirus of Gardner-Arnstein feline sarcoma virus. Proc. Natl. Acad. Sci. USA 78:3580-3584.

Moynet, D., and Garon, C. F. 1981. Streptococcal bacteriophage phi-42 DNA has a cyclic permutation and a terminal repetition. Virology 109:211-214.

LABORATORY OF CLINICAL INVESTIGATION
1981 Annual Report
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Summary of Program
Laboratory of Clinical Investigation
October 1, 1980 September 30, 1981

Michael M. Frank, M.D., Chief of Laboratory
and Clinical Director, NIAID

Introduction

The year of 1981 showed a number of important changes in the Laboratory of Clinical Investigation, however most important has been a general maturing of the program. No basic new elements have been added and there have been relatively few substantive changes. A very important organizational change was the development of a Laboratory of Immunoregulation under Dr. Anthony Fauci, and the departure of Dr. Fauci from the Laboratory of Clinical Investigation. Nevertheless, Dr. Fauci's unit and our unit continue to collaborate closely both in terms of clinical care, clinical investigation and basic scientific investigation. This organizational change recognizes the increasing international stature of Dr. Fauci without changing substantially the interactions of the clinical program. Dr. Fauci continues as Deputy Clinical Director of the program.

The Laboratory of Clinical Investigation continues to be divided into three basic areas--allergy, clinical immunology and infectious diseases. No new programs were introduced this year though it will be clear on inspection of the accomplishments of the various laboratories that each is assuming an increasingly important role in American biomedical and clinical investigation. The program in Humoral Immunity is under the direction of Dr. Frank. This program includes Dr. Carl Hammer, Miss Thelma Gaither as well as a number of clinical associates now termed medical staff fellows. This program is complemented by the Cellular Immunology program of the Laboratory of Immunoregulation. The Allergy Disease program continues under Dr. Michael Kaliner, as Chief of the Allergic Diseases Section. As discussed below under the development of the ACRF (Ambulatory Care Research Facility) we expect this section to undergo considerable development. Dr. Dean Metcalfe continues as a senior member of this section interested in the role of mucopolysaccharides in hypersensitivity and in the general area of food allergy. Dr. Ana Maria Saavedra-Delgado continues as a part time member of this unit and the unit has a number of visiting investigators as well.

The Infectious Disease group is represented by experts in each of the general areas of infectious disease. Dr. John Gallin and his group are responsible for studies of the phagocytic cell and its contribution to host defense. This group is particularly interested in patients with phagocytic cell defects and in patients with immunologic abnormalities and repeated infections such as those represented by the disease Job's Syndrome. Dr. John E. Bennett and Dr. June Kwon-Chung are responsible for the area of Clinical Mycology. Dr. Bennett is an internationally known clinician in this area and Dr. June Kwon-Chung, Ph.D. is an expert mycologist interested in biochemical characterization and genetic analysis of fungal strains.

The general area of Virology is represented by Dr. Stephen Straus who is responsible for our clinical viral program as well as for clinical investigation of virally transmitted disease and the development of anti viral agents. The area of Bacterial Disease is represented by the unit under Dr. Stephen Hosea whose major interest has been in the area of bacteremia and its consequences. This section has two additional clinical investigators with broad experience in the area of internal medicine, infectious disease and host defense: Dr. Eric Brown and Dr. Keith Joiner. Dr. Eric Brown was a clinical associate within the laboratory and was trained under Dr. Hosea and Dr. Frank in the area of host defense against bacterial infection. He has served in an increasingly independent capacity in the past years in becoming expert in studies of bacteremia and the mechanism of bacteria clearance from the bloodstream. Dr. Joiner is a recent addition to the laboratory who has come as a post-doctoral fellow interested in the interaction of complement with pathogenic bacteria. In mid summer, Dr. Hosea decided to leave the Laboratory of Clinical Investigation and pursue a new career in California. Dr. Brown and Dr. Joiner will continue the direction of that laboratory and major change is, at present, not forseen.

Thus, the three general areas of Clinical Immunology, Allergy and Infectious Disease are each represented by individuals who are internationally known for their expertise in clinical investigation as well as clinical care. The details of the programs under study will be enumerated below. Nevertheless it should be said that the senior staff has achieved wide recognition for the quality of its research efforts and the Laboratory of Clinical Investigation has come to be considered one of the strongest clinical investigation units in these areas in the country.

CLINICAL IMMUNOLOGY SECTION

The Clinical Immunology Section was developed to explore the humoral immune system and its contributions to host defense as well as its contributions to pathogenesis of disease. Two areas of investigation have been under study. The first, the role of complement in the production of host defense and immunologic damage, and the second, the mechanisms of immune complex disease and their role in the development of autoimmunity. There have been major advances made in both of these general areas. The work of the past three years in developing methods for the purification of human complement components suitable for both clinical and basic studies has continued in a most satisfactory manner. As will be clear below, the availability of these components is allowing for new and important insights to be gained in the mechanisms of complement action. It is hoped that the availability of these new reagents will in addition allow for the development of important new programs in the role of complement component function in man. Within the past year important new information has been gathered in mundane but vital areas such as methods for storage of these components and it has been shown that some components will have to be prepared at periodic intervals because of the intrinsic biochemical instability of the components. Thus, C3 and C4 have an internal thioester bond which upon hydrolysis leads to loss of biochemical activity. These components must be prepared at periodic intervals (Hammer, Gaither, Renfer). Previous studies had suggested that native C3 interacts with C3 receptors on lymphocytes and phagocytic cells, thus blocking the interaction of C3 on opsonized particles with those phagocytic cell receptors. Studies in the laboratory this year, using the new improved methods for the purification of C3 have shown conclusively this is not the case. Serum C3 does not block phagocytic cell receptors unless the C3 has been damaged during the purification procedure as was true for all other preparations of C3 reported in the literature. Thus, C3 prepared by the new method does not block these receptors and does not interfere with phagocytosis of opsonized particles. This important insight is key to understanding the relative roles of C3 and IgG Fc receptors on phagocytic cells. Since the particles to be phagocytized exist in medium which contains both complement and immunoglobulin it has been difficult to understand how phagocytosis proceeds when the receptors are blocked by the free immunoglobulin and C3 molecules. This work proves conclusively that although IgG receptors are blocked and therefore ineffective, C3 receptors remain effective even in the presence of high serum concentrations of C3 since they recognize a cleavage product of C3 and do not recognize native C3. (Berger, Hammer, Gaither and Frank). The cleavage of C3 has been under extensive investigation (Gaither, Hammer, Frank). It has been shown that neutrophil proteases are capable of cleaving the opsonically active C3b to its degradation products. Assays have been developed for studying this in detail and it has been shown that neutrophil elastase is the major enzyme responsible for this effect. Moreover, the cleavage has been shown to proceed in multiple steps. Since some of the degredation products of C3 interact in a very specific way with phagocytic cells, detailed knowledge of the degradation steps and their control is essential to understanding the phagocytic cell process in host defense. Other studies of the biochemistry of complement components have examined the distribution of complement components on cell surfaces as well as the distribution of receptors for these components. The importance of clustered vs. nonclustered complement components on the surface of opsonized particles has become key to our understanding of the processes which lead to phagocytosis as opposed to direct lysis of cells.

The role of complement and immunoglobulin receptors in normal physiologic processes continues to be studied. We reported in the past year with Drs. Lawley, Hall, Katz and Fauci that a defect exists in the clearance of IgG sensitized erythrocytes from the circulation of patients with dermatitis herpetiformus. In studying this defect more carefully it was shown that this defect relates not to the disease but to the fact that all of these patients have a particular HLA type (HLA B8 DrW3). It was shown that approximately one half of normal individuals with this HLA type have a similar defect at a time that they are considered perfectly normal and free of disease. The importance of this finding is being evaluated at present. It is known that this HLA type is associated with the development of autoimmune disease. One possible scenario is that patients with this HLA type have a defect in Fc receptor function and this defect leads to a defect in the clearance of immune complexes from the circulation. This in turn may lead to the build up of immune complexes in the circulation with their subsequent deposition in tissues.

As a further development of this observation it has become important to examine Fc receptor activity on peripheral blood cells in patients with a variety of diseases as well as in patients with the B8 haplotype. Two approaches in the laboratory by Drs. Inada and Fries suggested that, in fact, peripheral blood monocytes do not express an Fc receptor defect when examined directly. One of these approaches, which involves the study of direct binding of immunoglobulin to monocytes of these patients has been able to quantitate the number of binding sights as well as the average affinity constant in a large number of normal controls as well as patients. However, striking abnormalities in Fc receptor number and binding constant have been found in individuals with a variety of autoimmune diseases and these are now being explored in detail.

The interactions of complement components with bacteria have been examined in detail over the past year and a number of important observations have been made. In clinical studies it has been shown that patients with abnormal splenic function and splenectomy make poor responses to the pneumococcal vaccine in both the IgG and IgM classes of antibody (Hosea, Brown, Burch and Frank). This is terribly important because that vaccine is being promoted as an effective method of preventing the development of a penumococcal infection in that patient population. Assays have been developed in the laboratory which allow one to correlate quantitative antibody levels with bactericidal activity of serum for the first time, and for the first time an effective ELISA assay for anti pneumococcal antibody has been developed. This assay allows human IgM and IgG anti pneumococcal antibodies to be quantitated for the first time. It has been shown that the location of antibody and complement components on a bacterial surface play a critical role in determining the pathogenecity of the bacterium and its interaction with phagocytic cells. Thus, it has been shown that antipneumococcal cell wall antibody is non protective and non opsonic as compared to anticapsular antibody, even though it is capable of depositing large numbers of C3 and other complement component molecules on the surface of the bacterium. Presumably the complement deposition is in a site which cannot interact with receptors on phagocytic cell surfaces (Brown, Hosea and Frank).

Also this year an important breakthrough has been made in our understanding of the mechanisms by which some bacteria prove to be complement resistant and others complement sensitive. Although this work is still being completed it is clear that serum resistant bacteria activate complement at least as well as a serum sensitive bacteria. The resistant bacteria activate complement and assemble the macromolecular complement membrane attack complex. However, instead of successfully inserting into the bacterial cell membrane the attack complex is shed. Complement cleavage by the resistant bacteria is enhanced and destroys all of the effective opsonin available (Joiner, Brown and Frank).

CLINICAL MYCOLOGY SECTION

The Clinical Mycology Section continues to be interested in three general areas. The first is the diagnosis of fungal infections. This is a major clinical problem because fungal infections are frequently very subtle and hard to diagnose definitively in patients with host defense defects. Second is the therapy of fungal infections and third is factors in the metabolism in chemistry of the fungal organism which influence its pathogenesis. Both morphologic, taxonomic and epidemiologic studies are included in the section. Most of the emphasis of the section has centered on the Cryptococcus neoformans and within the past year further progress was made in the genetic classification of subgroups of the cryptococcus with different serotypic characteristics. The chemical basis for the serologic difference was further defined. Moreover, the biochemistry of the organisms, particularly with regard to the synthesis of melanin was characterized. Hybridization techniques were developed to test relatedness of various cryptococcal strains and it was possible to show the DNAs of serotypes B and C were closely related while those of A and D differed significantly from that of serotypes B and C. Moreover it was shown that certain biochemical characteristics of the cryptococcus relate to its virulence. In particular isolates lacking phenoloxidase are avirulent for mice. The biochemical basis for these virulence characteristics is under study at present.

It was also shown that patients who had cryptococcal disease and were thereafter cured, had longlasting specific decreased antibody responses to immunization with cryptococca polysaccharide. Further investigation will allow us to determine whether this represents specific immunologic unresponsiveness resulting from infection or precedes infection thereby predisposing the patient to cryptococcal infection.

Several other fungi were studied during the year. Studies of the Aspergillis fumigatus were conducted using radiolabelled antigenic fungal component galactomannan. This material was shown to appear in the urine of rabbits infected with this organism. In unimmunized animals the material is largely excreted in the urine though about 20 per cent was deposited in the liver. It is hoped that increasingly sensitive radioimmunoassays for these fungal products will allow for specific diagnoses to be made early in infection when currently available techniques preclude the diagnosis. The section also evaluated a number of the currently available tests in use for the diagnosis of Candida albicans infection. It showed that one test was best able to predict protection of infected mice by anti fungal agents and further showed that 11 to 35 per cent of Candida albicans from various centers were resistant to the drug flucytosine.

MEDICAL VIROLOGY SECTION

The Medical Virology Section has continued to expand its interests in a variety of viral diseases since Dr. Straus took over direction of the section several years ago. Over this period of time the section has seen the successful development of ELISA assays for influenza and adenovirus antigens and for detecting type specific pneumococcal antibody. Some of these tests are now in routine use in the Laboratory of Clinical Investigation. A second major area of interest of the section is in the clinical and biochemical studies of human Enteral adenovirus infections. Enteral adenoviruses are a recently discovered group of agents which cause enteritis in infants. They differ from all other known human adenoviruses in the inability to be propagated in tissue culture. During the past year the Medical Virology Section continued to obtain specimens from sources around the world representing epidemic diarrheal outbreaks. Stool specimens were characterized by enzyme immunoassay, electron microscopy, and propagation in tissue culture. The Enteral adeno viruses were studied extensively to determine the biologic basis for their inability to grow in tissue culture. It was learned that these agents induce an abortive infection which is abruptly terminated at a very early step of the virus growth cycle in vitro. Using this information Dr. Straus and colleagues developed a successful strategy to complement the block to virus replication and have succeeded for the first time in permiting the efficient replication of these viruses in tissue culture. Enteral adenoviruses grown in culture for the first time have now been analyzed and characterized extensively in terms of their polypeptides, the structure of their genomes, and their antigenicity.

The pathogenesis, molecular biology, immunology, natural history and therapy of human herpes virus infections has also been investigated in the Medical Virology Section. Immunocompetent and immunodeficient patients with a wide variety of herpes virus infections have been identified, followed clinically, diagnosed by virus isolation, studied immunologically and in a number of cases, treated successfully with systemic antiviral agents. Major success has been experienced in the use of the agent acyclovir for the treatment of herpes simplex virus. This agent appears to rapidly eliminate shedding a virus in the patient allowing healing of lesions. However it does not eliminate all sources of virus within the body. Thus the patient may relapse at some later time.

One major focus in the study of herpes virus has concerned the molecular biology and latency of varicella zoster virus. During the past year the group has completed studies on the restriction endonuclease analysis and molecular epidemiology of the varicella zoster virus DNA. They have prepared a library of varicella zoster DNA fragments using molecular cloning techniques and have come far in completing physical mapping of the varicella zoster virus genome. Hopefully they will be able to use this information in studies of the basis of latency, the characteristic of the virus which allows it to exist within cells protected from the host defense mechanism and able to cause reinfection in patients even though they have specific immunity.

BACTERIAL DISEASES SECTION

The Bacterial Diseases Section has its major interest in the function of the phagocytic cell, its mechanism of action, the biochemistry of events which take place within the cell membrane, and the biological and clinical consequences of abnormal phagocytic cell function. Many new findings have appeared within the past year which are detailed in the section's report. Only a few of these will be summarized here. Major technical advances were made using newly developed equipment for quantitating leukocyte chemotaxis, bactericidal activity, and availability of Fc and C3b membrane receptors. Using an Optomax image analyzer, the amount of time required to quantitate the number of cells migrating a distance into a millipore filter for chemotactic assays has been reduced by 90 per cent. Moreover the accuracy of the technique has been increased enormously. Micro methods have been developed for determining in a quantitative way cell spreading as well as cell rosette formation. These techniques will allow more careful analysis of patient samples as well as the analysis of samples that come from children--something that has presented great difficulty in the past.

The quantitative relationship between binding of chemotactic stimuli and secretagogues has been studied extensively within the past year, as has the relation between granule associated receptors and cell membrane associated receptors and their interaction. The section is in the process of developing a generalized theory for the turnover of receptors and their mechanism of action which is potentially an extremely important issue. As part of this they had the opportunity to study in detail a patient whose neutrophils were missing specific granules. Studies in this patient included the demonstration of decreased binding of specific chemoattractants as well as a defect in receptor mobilization following stimulation of the cells. The relationship between stimulation of cell surface receptors and behavior of the cells in terms of increased adhesiveness, margination, and ultimately chemotaxis are under evaluation as a long-term project of the laboratory. It is now possible to quantitate the number of receptors for certain materials such as the chemotactic peptide f-met-leu-phe. The data show that chemotactic deactivation accomplished with this peptide leads to a loss of receptors for this peptide. When the deactivation is accomplished with an unrelated chemotactic peptide the actual number of receptors increases.

An important series of experiments was initiated examining the effect of vitamin K derivatives on neutrophil function. It was shown that these vitamins are effective inhibitors of neutrophil spreading, locomotion, phagocytosis and killing without effecting receptor availability, HMP shunt activity or lactate production. Studies suggest that vitamin K exerts its effect via influencing the movement of calcium across membranes and the sequestration of calcium within the cell, as well as other biochemical events within the cell such as the tyrosylation of the alpha chain of tubulin. The section has had a major interest in the use of biochemical probes to study cell membrane potential and has developed new probes with increased potential usefulness over the past year. Using similar technology it has been shown to be possible to study the effect of certain antibiotics on bacterial cell membranes which may lead to further understanding of bacterial susceptibility to antibiotics. Such fluorescent probes have also allowed for the demonstration of additional subsets of neutrophils some of which respond to these probes and some of which do not. The whole area of subsets of neutrophils has been of major interest to the section for a number of years, because it appears that there are subsets of neutrophils which respond to infectious challenge and others which do not. The relationships of these subsets may be of key importance in determining the outcome of certain infectious diseases.

New tests have been developed for the diagnosis of patients with chronic granulomanus disease and for the detection of carriers of this disease. This may have major therapeutic and diagnostic implications in this unfortunate group of patients. A second interesting group of patients with chemotactic defects are those with the Chediak-Higashi syndrome. The abnormality in PMN function was shown to be associated with the 300 fold increase in the tyrosylation of the alpha chain of tubulin. This represents the first biochemical evidence for abnormal microtubule metabolism in a patient with a phagocytic cell dysfunction.

Other clinical states have also been under study. Pregnancy is associated with an increase in a number of circulating neutrophils. Members of this section have shown within the past year that neutrophils obtained from pregnant individuals have marked alterations in their receptor numbers, their ability to generate certain toxic products such as superoxide and well as other functions. Such alterations may contribute to the increased mortality from certain infections seen during pregnancy and may explain some of the features that characterize the development of intrauterine infection. Another disease under study has been that associated with hyperimmunoglobulin E and recurrent infection syndrome. A prospective double blind study of the clinical efficacy of a drug levamisole is underway. It was also shown that the mononuclear cells of these patients produce a factor which inhibits normal neutrophil chemotaxis. Obviously this may be very important in the development of infection in these patients. In all of these studies both clinical observations as well as basic scientific analysis are being utilized to determine why certain groups of patients are susceptible to infection and others are not. Although this area is in its infancy still at present it is of major import in the large number of patients with frequent infections.

ALLERGIC DISEASES SECTION

The Allergic Diseases Section has been very active in the past year attempting to expand knowledge in all areas of immediate hypersensitivity function. The particular emphasis of the section has been in defining the biochemical events which accompany and control the triggering of allergic reactions, the neurophysiologic mechanisms which potentially modulate these reactions, the characterization of the mediators of anaphylaxis and the identification of the responses of relevant target tissues to those mediators. To this end a large number of different systems are under study. These include human systems where chopped human lung fragments, obtained from patients undergoing surgical resection for other diseases, are examined for their release of mediators and mucous. Also studies are performed both in a rat model as well as with peripheral blood cells to determine various facets of immediate hypersensitivity function. Major findings are listed in the specific summary of the Allergic Disease Section projects. Nevertheless it should be mentioned that great progress has been made in understanding mucous secretion and the factors which govern mucous secretion in the human lung. Since mucous secretion is a prime component of the development of the asthmatic attack, it is of key importance in determining asthma therapy. It has been shown that a class of endogenous synthesized chemical substances, monohydroxyeicosatetraenoic acids, increase mucous secretion enormously at low concentrations. These substances can be released during immediate hypersensitivity reactions. Moreover, slow reacting substance of analphylaxis, long thought to be a major mediator of asthma, also increases mucous secretion. Glucocorticoids inhibit mucous secretion in a dose related fashion primarily by decreasing the baseline mucous secretion of cells.

In immediate hypersensitivity reaction it is known that there is an early urticarial phase as well as a late infiltrative phase of the reaction. This late infiltrative phase may clinically be important as in patients with late phase bronchoconstriction following allergic challenge of their airways. The basis of this has been under study in the section for a number of years and the relationship between the release of mast cell granular substance and the formation of these reactions is under specific examination. The importance of neutrophils in the development of the late phase response has now been established and the level of peripheral eosinophilia was found to be unimportant in the extent of infiltration.

This section has had a major interest in studying β adrenergic receptors as well as histamine receptors on cells. It has been possible to characterize cell membrane as well as granule beta adrenergic receptors and show that mast cell granule membranes as well as cell membranes have a similar high affinity receptor but the cell membranes also having a low affinity high capacity receptor. The role of these receptors in biologic function is under study. In a similar way histamine receptors on these same cells are under extensive analysis. Those studies suggest that mast cells have an H1 receptor which is linked to the adenylcyclase system but this linkage has not been shown for H2 receptors. Since opiates are known to cause mast cell degranulation in man, mast cells have been examined for the presence of opiate receptors. These receptors have been identified and are currently being characterized on human mast cells, although it has been difficult or impossible to demonstrate such receptors on rat mast cells.

In clinical studies the response of normal volunteers to histamine infusion has been carefully evaluated. Histamine infused intravenously causes flushing, headaches, tachy-cardia and hypotension. Pretreatment of subjects with H1 and/or H2 antihistamines was used to determine which receptors were involved in each response. Both H1 and H2 receptor antagonists were required to prevent flushing, headaches and hypotension. H1 receptor antagonists alone reduced the tachycardia, but H1 and H2 antagonists in combination were even more effective. Tachycardia appeared to be the most sensitive clinical parameter to histamine infusion and appeared at 1.5 to 2 nanograms of histamine per ml of plasma. Assays of histamine, particularly in the urine, have been frought with difficulty and during the past year this section has developed new and sensitive assays for the measurement of urinary histamines. Using these assays a wide variety of clinical illnesses are now being explored for secretion for urinary histamine.

Dr. Metcalfe's group in the Allergic Diseases Section has continued its studies of proteoglycans in mast cells. A proteoglycan heparin has been identified and characterized in mouse mast cells grown in vitro in lymphocyte dependent culture systems. A histamine containing cell cloned from mouse bone marrow was identified as an atypical mast cell containing a unique proteoglycan, chondroitin 4-sulfate. The function of heparin may be in part to form a protease resistant heparin granule which persists in tissues after release from mast cells until phagocytosed by connective tissue fibroblasts. This phagocytosis of mast cell granules has been carefully documented in vitro in the section this year. Following phagocytosis of these heparin containing granules, fibroblasts alter their metabolism and respond with increased enzyme secretion. As part of its interest in mast cells the section has begun to accumulate a group of patients with systemic mastocytosis. Clinical evaluation of these patients has already revealed a number of new findings. It has been shown that these patients have a high incidence of type IV hyperlipoproteinemia as well as bone marrow and cardiac disease. Many of these patients have mitral prolapse. Thus, the section is making strides both in the basic science of immediate hypersensitivity as well as in quite highly applied areas of allergy investigation related to control or amelioration of symptoms in patient groups.

CLINICAL PARASITOLOGY SECTION

Although a variety of parasitologic problems and questions were seen by the Clinical Parasitology Section in the past year, most research has concentrated upon leishmaniasis, schistosomiasis and filariasis. The diffuse cutaneous leishmaniasis (DCL) patients studied at NIH the past year were followed up in the Dominican Republic. At 6 months all were well, but at one year one of the amphotericin treated cases had relapsed. In the endemic area individuals without evidence of current or past leishmaniasis were found skin test positive to leishmanin antigen. In contrast to the DCL patients, such skin test positive cases generally showed blastogenic response to leishmanial antigen. The reason for such a large proportion of DCL cases in this region is not known, but the parasite strain does appear to be unique. One new case and one drug-relapse case were brought to the NIH for local heat treatment, with good immediate results. Depression of immediate hypersensitivity and cell-mediated reactions that are found in patients with chronic schistosomiasis was reversed after treatment of their infections. The development of assays for antibodies to a variety of parasite antigens promises to be useful for both diagnosis as well as better understanding of pathophysiology of certain parasitic infections. It is now possible to measure parasite-specific IgE to schistosome and filarial antigens, including different stages of the parasites. Further observations on the antibody response to two carbohydrate antigens from schistosome worms have confirmed the usefulness of these tests to determine duration and degree of human infection with this parasite. An ELISA test, using larval antigens, was developed and found to detect antibodies in 80 to 85 per cent of parasitologically proven human strongyloides infections. A special protocol permitting use of a new anti-schistosomal drug, Praziquantel, was developed for treatment of S. mekongi infections in Laotian refugees. Results of treatment were excellent and side-effects were minimal.

ADMINISTRATIVE AND ORGANIZATIONAL CHANGES

As mentioned in the Introduction there have been a number of important organizational and administrative changes during the past year. Dr. Anthony Fauci's Cellular Immunology unit was separated from the Laboratory of Clinical Investigation to form a separate laboratory, The Laboratory of Immunoregulation. Dr. Fauci continues as Deputy Clinical Director of the NIAID.

There were no major changes in the other Sections of the NIAID with one exception. Dr. Stephen Hosea, a member of the Clinical Immunology Section and an expert in bacterial mediated infectious diseases left the Institute this year. His critical position within the Laboratory will be filled by Dr. Eric Brown, a graduate of Harvard College and Harvard Medical School, who is fully trained in Internal Medicine and is Board Certified in Internal Medicine and in Infectious Diseases. Dr. Brown will continue the interests in host defense against bacterial disease that he developed working with Drs. Frank and Hosea. Within the past year Dr. Brown's efforts have been aided by Dr. Keith Joiner, another young Internist with Boards in both Internal Medicine and Infectious Disease who has joined the Laboratory to study host defense mechanisms against bacterial infection.

Thus, the number of changes within the past year has been small. Dr. Fauci's group, although no longer part of the Laboratory of Clinical Investigation, continues to act as it did before the organizational change. Dr. Hosea, although his presence will be missed, had trained other members of the unit effectively to take his place.

The major change, awaited eagerly by the staff of the Laboratory of Clinical Investigation as these words are being written, is the imminent opening of the Ambulatory Care Research Facility, the ACRF. It is hoped that the facility will open during the summer of 1981. In the Facility will be an entirely new Outpatient Department which will allow us to greatly expand our current capabilities in that area as well as about 2800 square feet of additional laboratory space. It is planned that Dr. Kaliner's allergy group will move into the ACRF as well as Dr. Ottesen's Parasitology unit. This will represent the major new space available in the Institute within the past decade and is awaited with great anticipation.

HONORS AND AWARDS

The members of the Laboratory of Clinical Investigation have received considerable recognition during the past year. This recognition is exemplified by invitations to become members of the editorial boards of many of our leading journals. Thus, the Journal of Clinical Investigation has two members of the Laboratory of Clinical Investigation on its editorial committee: Dr. Michael Frank and Dr. John Gallin. Dr. Gallin also serves on the editorial board of the Journal of Immunology. Dr. Frank and Dr. Kaliner serve on the editorial board for the official journal of the American Academy of Allergy--the Journal of Allergy and Clinical Immunology. Dr. Bennett serves on the editorial board of the Journal of Infectious Disease and Dr. Frank serves on the editorial board of Reviews in Infectious Diseases. Dr. Frank serves on the editorial board of the official journal of the American Society for Hematology--Blood. Dr. Frank continues as Secretary-Treasurer of the American Society for Clinical Investigation and is a member of the organizing committee of the Complement Workshop. Dr. Gallin serves on the editorial board of the journal--Inflammation.

This year, Dr. Kaliner was invited to a six year term as a member of the Board in Allergy and Clinical Immunology. This is a conjoined board of the American Board of Internal Medicine and the American Board of Pediatrics. Moreover, within the past year, Dr. June Kwon-Chung was selected as the second Maxwell Littman Lecturer for the New York Society of Medical Mycology. Dr. Bennett was elected a member of the Infectious Disease Committee of the American College of Physicians and a member of the Medical Research Science Merit Review Board of the Veteran's Administration. Dr. Frank was elected a Director of the Foundation for Advanced Education in the Sciences.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00043-16 LCI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunology and Chemotherapy of Systemic Mycoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.E. Bennett	Head, Clinical Mycology Section	LCI NIAID
OTHER:	Bertrand Dupont	Guest Worker	" "
	K.J. Kwon-Chung	Senior Investigator	" "

COOPERATING UNITS (if any)
D. Stevens and R. Stiller, Stanford Univ.; H. Scholer, Basle, Switzerland;
D. Henderson, CC, NIH

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Mycology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Aspergillus galactomannan appeared in urine of rabbits infected with A. fumigatus. Radiolabeled galactomannan was largely excreted in urine (42%) or deposited in liver (20%) of unimmunized rabbits.

2. Cured cryptococcosis patients had long-lasting, specific, decreased antibody response to immunization with cryptococcal polysaccharide.

3. The in vitro susceptibility test best able to predict protection of infected mice found that 11.5-35% of Candida albicans from various medical centers were resistant to flucytosine.

Project Description:

The general goals of the unit have been to study host defense in systemic mycoses and to improve diagnosis and treatment of these disorders.

Objectives:

1) Investigate B cell tolerance in human cryptococcosis.

2) Develop diagnostic test for invasive aspergillosis.

3) Assess *in vivo* correlation of *in vitro* tests for flucytosine susceptibility.

Methodology, Results and Significance:

1) At time of diagnosis, 70% of patients with cryptococcal meningitis have cryptococcal polysaccharide (CPS) in their serum. Among 53 cured patients followed for at least one year, type specific serum antibody to CPS failed to appear in 60%. To test whether antibody negative patients were tolerant to CPS, 8 patients with prior cryptococcal meningitis and no serious underlying disease were studied an average of 5.5 years following completion of therapy. These patients had been free of serum and CSF antigen for an average of 3.9 years. Patients were vaccinated subcutaneously with 37 μg CPS and 50 μg type III pneumococcal polysaccharide (SSS III). Ten normal volunteers were also vaccinated. Blood was obtained immediately before and two weeks following vaccination. Antibody was measured by a Farr-type RIA, sensitive to 27 ng antibody/ml. Geometric mean titers to CPS were 1:1 in both groups before vaccination, but were 1:3 in patients and 1:119 in controls post-immunization ($p<0.01$, student's t test). Class-specific antibody to CPS, measured by ELISA, found little IgG or IgA but found geometric mean post-vaccination IgM titers of 1:31 in patients and 1:238 in controls ($p<0.01$). Responses to SSS III were similar in patients and controls, with IgA, IgM and IgG mean titers of 1:1129, 1:369 and 1:158 in patients and 1:1504, 1:1039 and 1:163 in controls ($p>0.2$ for each antibody class).

Cured cryptococcal meningitis is often associated with prolonged, specific tolerance to CPS, even in previously normal patients. Immunologic unresponsiveness in these patients may be pre-existent or may be antigen-induced during illness. (Bennett, Henderson)

2) Considerable discrepancy exists between laboratories concerning the frequency of resistance to the antifungal drug, flucytosine, among isolates of *Candida albicans*. In a collaborative study, 400 *C. albicans* isolates, obtained from five widely separated centers, have been tested for flucytosine susceptibility *in vitro* and *in vivo*. Results of three different *in vitro* tests were interpreted to mean that each isolate had several different subpopulations which varied in flucytosine susceptibility. This heterogeneity was thought to reflect heterozygosity in this diploid species. Tube dilution susceptibility testing using 48 hours of incubation correlated best with *in vivo* protection in mice given experimental candidiasis, probably because both this *in vitro* test and the animal model measured drug sensitivity of the major, but not minor, fungal subpopulations. With the 48-hour tube dilution test, drug resistance

was found in 11.5-15.5% of isolates in four centers and 35% in the fifth. Serotype B isolates accounted for 42% of all susceptible isolates but 81% of the resistant isolates, indicating linkage between these two markers. Induction of homozygous mutants with mutagens may help elucidate the genetic basis of drug resistance and linkage to serotype. (Bennett, Stevens, Stiller, Scholer)

3) Interest in rapid diagnosis of aspergillosis in the immunosuppressed patient has led to attempts at diagnosis by antigen detection. ELISA inhibition assay detected Aspergillus fumigatus galactomannan at concentrations of 40-2000 ng/ml in urine of all lethally infected rabbits as well as in the urine of some rabbits receiving a slightly lower than lethal inoculum. Catabolism of ^{125}I-tyraminyl AS$_{II}$ was studied following intravenous injection into rabbits. In normal rabbits, approximately half the antigen was excreted rapidly into the urine and a fifth is deposited in the liver. Urine excretion of antigen was markedly reduced in immunized rabbits, more antigen accumulated in liver and spleen and more of the serum and urine isotope no longer bound to antibody. Considering that normal volunteers have antibody to Aspergillus galactomannan by radioimmunoassay, the immunized rabbit model may be relevant to the infected patient. Future studies will investigate the possibility that galactomannan attached to the galactosyl receptor of hepatic Kupffer cells. In addition, purified polysaccharide has been provided to Dr. Bhattacharjee for structural analysis. (Bennett, Dupont)

4) Serotype of capsular polysaccharide in Cryptococcus neoformans was found to correlate with release of ammonia from glycine. A simple culture medium was devised to separate serotypes B-C from A-D. (Kwon-Chung and Bennett)

Publications:

1. Bennett, J.E.: Chapters - General Considerations (infections caused by higher bacteria and fungi), Actinomycosis, Nocardiosis, Cryptococcosis, Blastomycosis, Histoplasmosis, Coccidioidomycosis, Sporotrichosis, Moniliasis (Candidiasis), Aspergillosis, Phycomycosis, Other Deep Mycoses, and Superficial Mycoses Including Ringworm. In Isselbacher, K.J., Adams, R.D., Braunwald, E., Petersdorf, R.G., Wilson, J.D. (Eds.): Harrisons Principles of Internal Medicine, 9th edition, New York, N.Y., McGraw Hill, 1980, chapters 150-162, pp 733-746.

2. Bennett, J.E.: Cryptococcosis. Editorial in Western J. Med. Vol. 132, No. 2, p 150, 1980.

3. Dismukes, W.E., Bennett, J.E., Drutz, D.J., Graybill, J.R., Remington, J.S., Stevens, D.A.: Criteria for the clinical evaluation of antifungal drugs. Rev. Infect. Dis. Vol. 2, No. 4, 535-545, 1980.

4. Bennett, J.E.: Treatment of cryptococcal, candidal and coccidioidal meningitis. In Remington, J.S. and Swartz, M. (Eds.): Current Clinical Topics in Infectious Disease, Vol. 2. New York, McGraw Hill, 1980, pp 54-67.

5. Kwon-Chung, K.J., Hill, W.B. and Bennett, J.E.: A new special stain for the histopathological diagnosis of cryptococcosis. J. Clin. Microbiol. 13:383-387, 1981.

6. Bennett, J.E.: Cryptococcal skin test antigen: preparation variables and characterization. Infect. Immun. 32:373-380, 1981.

7. Bennett, J.E. and Remington, J.S.: Miconazole in cryptococcosis and systemic candidiasis: a word of caution. Ann. Intern. Med. 94:708-709, 1981 (editorial).

8. Bennett, J.E.: Systemic Mycoses. In Cluff, L.E. and Johnson, J.E., III (Eds.): Clinical Concepts in Infectious Diseases, 3rd edition. Baltimore, Md., Williams & Wilkins. In press.

9. Bennett, J.E.: Cryptococcal meningitis and other mycoses of the central nervous system in cancer patients. In Klastersky, J. (Ed.): Non-bacterial Infections in Cancer Patients, New York, Raven Press. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 A1 00045-13 LCI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Studies on the Interaction of Antibody and Complement on the Production of Immune Damage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	T. Gaither	Research Biologist	LCI	NIAID
	L. Renfer	Chemist	LCI	NIAID
	H. Gresham	Med. Tech.	LCI	NIAID
	E. Brown	Medical Officer, CO	LCI	NIAID
	S. Hosea	Senior Investigator	LCI	NIAID
	K. Joiner	Medical Officer, CO	LCI	NIAID
	C. Burch	Biologist	LCI	NIAID
	C. Brickman	Clinical Associate	LCI	NIAID
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Progress has been made in the purification and characterization of complement components and in studies of their mechanism of interaction. We have shown that intact C3 does not bind to receptors, while C3b does bind. These findings have been made possible by the new methods of purification of complement components which leave them in the native rather than altered state. The regulation of biologically active fragments of C3 by cells and their products has been demonstrated. Major progress has also been made in the study of complement-bacterial interactions. It has been shown that human anti-pneumococcal antibody previously thought not to interact with complement does so and provides a measure of host defense. Failure of patients with splenectomy to synthesize antibody following pneumovax vaccination was documented. This required development of a new antibody assay procedure. The factors that contribute to the removal of bacteria from the bloodstream were documented in molecular terms. Anti-cell wall antibodies bind to bacteria and deposit complement molecules on the bacterial surface but provide no measure of host defense. Anti-pneumococcal capsule antibodies functioning in the same way provide specific increases in host defense capacity. The difference between susceptible and resistant bacteria to the lytic action of complement has been clarified.

Project Description:

Objectives:

The overall objectives of this project are to determine how the complement system functions in biochemical terms and to determine the biological effects produced via activation of this system. As a further extension of these studies the role of the complement system in the host defense mechanism and in the production of tissue damage in man will be evaluated. This is a long term goal and will be met via the completion of an extensive series of steps. These are detailed below.

Methods Employed:

A wide variety of biochemical and physiological techniques are employed. These include column chromatography of large batches of human and animal serum and plasma, using a wide variety of resins to separate proteins on the basis of charge, size, isoelectric point as well as more specific functions. These cover the range from column chromatography on DEAE-Sepharose to specific absorption chromatography using known immuno-absorbents. A wide variety of detailed functional assays are employed which measure the ability of individual complement components and complement component fragments to cause lysis of various indicator cells as well as to interact with specific receptors for those fragments. Techniques are developed to purify complement components to homogeneity and to cleave those components to known fragments of determined structure. Animal studies are employed to determine the biological activity of the fragments as well as their mechanism of action. New assays are developed to detect the presence of fragments in biological fluids as well as in patient materials. All of the various analytical methods including isoelectric focusing, PAGE electrophoresis, immunoelectrophoresis etc., are employed. Methods are developed to detect antibody at low concentration including ELISA techniques for the detection of antibodies to a variety of substances including specific bacterial antigens. Assays are developed to determine opsonization as well as bactericidal effects of antibody and complement on bacteria.

Major Findings:

We recently reported on the development of a large scale purification and isolation procedure for complement components C3, C5, C7 and C8 in biologically active and homogeneous form from one pool of human plasma. Other complement components (C1q, C4 binding protein, C3bINA, fB, C2, C6, B1H, C1EI and C9) are also obtainable in varying degrees of functional and biochemical purity. Additionally, we now have identified the elution positions of C1s and properdin and obtained these components in a relatively pure form. The properdin has been shown to be fully active as judged by its capacity to reconstitute properdin deficient serum in the killing of *T. vaginalis*.

Further purification of C4, B1H, C9 and C1EI has produced homogeneous preparations of C4, B1H and C1EI while C9 requires some additional effort. Our reported purification for C5 initially requiring an hydroxyl appatite adsorption step has been amended and is now performed utilizing an immunoadsorption procedure to remove contaminants more efficiently. This latter procedure results in C5 with improved recovery and specific functional activity.

Of all components, the third component of complement, C3, has a centrally important role in both the control of bacterial infection and in the pathophysiology of autoimmune disease. Consequently, efficient methods are required to isolate this protein as well as others in the system in a pure state and with lasting biological activity to enable a thorough investigation of their specific functions. With the known extreme lability of both complement components C3 and C4 further methodological alterations in our complement purification scheme have been incorporated to allow for improvements in the speed of isolation, recovery and consequently the final state of the purified products. The C3 from this latter procedure has full biological activity with better than 90% recovery as judged by immunological and functional criteria (Hammer, Renfer, Gresham and Frank).

We have found conditions which allow for storage of C3 in a pure state for at least 4 months at 4°C while retaining good activity. C4, however, is still labile under conditions of storage found optimal for C3. We have been able to extend the shelf life of C4 to at least seven months at 70°C but only in the presence of high concentrations of carrier protein which limits its usefulness.

Several investigators in our laboratory are studying bacterial-complement interactions in a guinea pig model system. Thus, purified guinea pig complement components have become indispensible. Application of the modified complement purification procedure to guinea pig plasma allowed for isolation of guinea pig C3 as a pure fully active protein with better than 90% recovery of functional activity (Hammer, Brown).

Studies on the mode of activation of the terminal complement components C5-C9 and their interaction and damage to biological membranes (erythrocyte membranes in particular) have shown that the lytic process is initiated when C5 is cleaved into C5a and C5b by the convertases generated either by the classical or alternative pathway. Another pathway of C5-9 activation in serum initiated by low pH has been recently reported (Hansch, Hammer, Shin). The active principle has been shown to be a product of C5 and C6 association designated $C(56)^{a}$ may be of biological import in both pathophysiology of infection and in autoimmune disease. Work is currently in progress on the physico-chemical characterization of the $C(56)^{a}$ lytic principle.

Paroxysmal nocturnal hemoglobinuria (PNH) is a disease characterized by an abnormal sensitivity of erythrocytes (PNH-E) to complement mediated lysis. A test described by Ham in which PNH-E, but not normal E (NHE) are lysed by acidified human serum is in general use but is poorly understood. In light of the properties of $C(56)^{a}$ and studies we recently performed we believe that acid activation of normal human serum occurs in the absence of C3 and alternative pathway activation to the same extent as in C3 replete serum. The acid activated $C(56)^{a}$ appears to be the lytic principle in acidified human serum (Hammer, Hansch, Shin).

The C5b fragment upon association with C6 forms a stable complex C5b,6 that lyses erythrocytes (E) together with C7,C8, and C9. We have now shown that the efficiency of lysis of differing species E depends primarily on the species of C8 and C9 but mostly attributable to C9. Lysis is least efficient when C9 is homologous with respect to target cell species and is not due to a failure to bind C9 but may be attributed to inefficient insertion and/or channel formation. (Hammer, Shin and Hansch).

Human C3 prepared by the methods recently developed by Dr. Hammer has been shown to fully retain the specific hemolytic activity of C3 in fresh serum. We have demonstrated that this C3 has only minimal ability to bind to C3b receptors of human cells even at 5x serum concentrations. C3b generated from this highly purified C3 by trypsin treatment effectively blocks the C3b receptors at 5×10^{8} M in rosette inhibition assays (Berger, Hammer, Frank). Thus native serum C3 does not block the C3b receptor unlike native serum IgG which blocks the IgG Fc receptor. The ability of particles opsonized with C3b to interact with the C3b receptor in the presence of serum C3 explain the ability of activated C3 on a foreign substance to remain on efficient opsonin in the presence of serum. This is in marked contrast to the IgG Fc receptor which operates inefficiently in the presence of serum IgG. We have found that C3 which has lost hemolytic activity on prolonged storage, after chaotrope treatment or after nucleophilic attack on its internal thioester bond has increased affinity for C3b receptors. These results may explain previous observations by others suggesting that native C3 as well as C3b bind equally well to the receptor. Thus, partially denatured C3, formed during C3 purification, interacted with the C3b receptor causing earlier investigators to believe that native C3 showed binding.

Human C3 has been biotinylated under mild conditions with excellent retention of hemolytic activity. The stochiometry of this reaction using biotinyl-N-hydroxysuccinimide has been studied, and incorporation of biotin into the C3d and C3c fragments has been documented. The high affinity of avidin for biotin ($K_A = 10^{15}$ M) can now be employed for the study of C3; cell bound C3 can be quantitated using fluorescein-avidin and flow cytometry and the distribution of C3 on the cell surface can be visualized in the electron microscope using ferritin-avidin. Dr. Thomas Chused and Dr. Roger Cole, respectively, have collaborated in these studies. Presently, we are using avidin-sepharose chromatography to identify and isolate the bacterial membrane constituents to which C3b becomes attached under a variety of conditions (Berger, Hammer, Brown, Joiner, Gaither).

The effect of proteolytic enzymes associated with human neutrophils on complement function is under study. One aspect of these studies has examined the cleavage of the third complement component, C3 (Gaither, Hammer, Frank). In these studies, a novel system was utilized in which proteolytic cleavage of two cell-bound C3 fragments, termed C3b and C3bi, was determined by measuring release of ^{125}I-C3 from complement coated red cells upon incubation with neutrophils. We were able to show PMN proteases released into the medium as well as PMN-surface associated proteases caused C3 cleavage. By utilizing specific protease inhibitors, it was demonstrated that elastase was primarily responsible for C3 cleavage.

The effect of elastase and other PMN proteases under more physiological conditions (i.e., in the presence of serum) is currently under study. The results of experiments in which ^{125}I-C3 release was measured in the presence of normal serum or α1 anti-trypsin deficient serum showed that inhibitors in normal serum do in fact regulate PMN cleavage of C3b and C3bi. The deficient serum, on the other hand, was significantly less effective in blocking ^{125}I-C3 release.

The distribution of C3b, which is the major opsonic fragment of complement, on the surface of a particle may influence the fate of the particle in vivo. An attempt was made to control C3b distribution on the surface of red cell intermediates by varying the input of purified early components of complement. The objective was to study surfaces on which C3b was distributed in "clusters" or "randomly". By using ^{125}I-C3 the number of molecules bound was readily determined and biological and hemolytic functional activity were also assessed. Preliminary findings indicated that C3b distribution may be important in that C3b which was presumably in "clusters" participated equally as well as "random" C3b in rosette formation with PMN but was only 1/10 as effective in forming a hemolytic C3 site (Gaither, Hammer, Frank).

We have participated in collaborative studies to determine the presence of complement receptors on various lymphoblastoid cell lines (Gaither, McGrath, Hammer, Frank). Currently, we are involved in a detailed study of receptors for cell bound complement fragments C3b, C3bi, C3d and C4 on Raji cells, which are tumor cells derived from B lymphocytes. There has been a great deal of controversy with regard to the presence of a C3b receptor on these cells and on the ability of these cells to synthesize complement control proteins which effect the expression of receptor activity.

By utilizing cellular intermediates to which very high concentrations of C3 and C4 were bound, we were able to demonstrate C3b receptor activity but not C4b activity. We also showed that soluble C3b blocked rosette expression with the C3b coated cells while soluble C4b failed to do so. These findings indicate that Raji cells have a receptor for C3b that is different from the C3b receptor on normal B lymphocytes which binds both C3b and C4b. We plan to confirm these findings by using specific antibodies to the C3b receptor which have been recently developed in other laboratories (Gaither, McGrath, Frank).

Immunization of normal volunteers with the polyvalent pneumococcal vaccine results in the elaboration of both IgG and IgM antipolysaccharide antibodies as measured by a specific ELISA assay. Efficient phagocytosis of pneumococci by polymorphonuclear leukocytes is critically dependent upon the amount of type specific IgG antibody in the sera and the ability of this antibody to activate the classical pathway of complement. A correlation between type specific antibody and bactericidal antibody titer had not previously been noted. Moreover prior workers using less sensitive techniques had come to the conclusion that human anti pneumococcal antibodies do not activate complement. This was shown to be incorrect (Hosea, Brown, Frank).

Pneumococcal polysaccharides excreted into solution can neutralize the opsonic effect of anti-pneumococcal antibodies. In addition, the polysaccharide capsule may block interaction of C3 bound at the surface of the organism with C3 receptors on polymorphonuclear leukocytes. Antibody directed against the polysaccharide activates C3 which may bind at the surface of the capsule and thereby allow interaction with polymorphonuclear leukocytes (Hosea, Brown, Frank).

After immunization extremely low levels of type specific anti-pneumococcal antibody were found in patients with high risk or history of pneumococcal infections. These include patients with splenectomy, sickle cell disease, hemodialysis and lupus. It may be that it is actually this inability to form antibody to polysaccharide antigens which predisposes these patients to pneumococcal infections. In patients with hypogammaglobulinemia on replacement therapy, levels of type specific antibodies were significantly higher than in those high risk patients after immunization (Hosea, Burch, Brolon, Frank). Most assays of response to the pneumococcal vaccine do not measure IgG antibody specifically which our studies suggest is the most important antibody class. In addition, patients at high risk of infection by virtue of their inability to make type specific IgG antibody could potentially benefit from gamma globulin replacement therapy. We have been engaged in a careful evaluation of the role of antibody and complement in host defense against pneumococcal bacteremia. As a first step, animal model of pneumococcal bacteremia was developed (Hosea, Brown, Frank).

We have made major use of a guinea pig model of pneumococcal bacteremia. We have also used purified rabbit antipneumococcal antibodies both IgG and IgM. We have used purified human complement proteins provided by Dr. Carl Hammer and have also with his help adapted his method for purifying human complement proteins to the purification of guinea pig C3 and C5. We have used purified bovine conglutinin in a radio-binding assay for the detection of surface bound C3bi.

We have also spent some time developing techniques for radiolabelling live pneumococci and have extensively employed a completely defined medium for pneumococcal growth originally developed by Dr. Alexander Tomasz of the Rockefeller University for biological radiolabelling of pneumococci with ^{75}Se, ^{59}Fe or ^{3}H-choline.

We have used the experimental model of pneumococcal bacteremia in the guinea pig to make the following observations: First, normal kinetics of clearance of bacteremia in non-immune or immune animals requires complement activation (Hosea, Brown, Frank). Second, complement activation is required for normal hepatic sequestration of pneumococci (Brown, Hosea, Frank). Third, clearance kinetics, RES localization, and opsonization requirements are dependent on the phase of the growth cycle from which the injected pneumococci are taken (Brown, Hosea, Frank). Fourth, that the role of the spleen in clearance of pneumococci is to sequester more virulent or less well opsonized organisms (Brown, Hosea, Frank). Fifth, that purified anticapsular antibody (either IgG or IgM) requires complement activation for an in vivo effect, and that as little as 100 IgM or 1400 IgG per cfu can induce significant improvements in the rate of pneumococcal clearance (Brown, Hosea, Frank).

These studies have been extended *in vivo* and *in vitro* to compare the complement activating and opsonic effects of antibodies directed either against the pneumococcal capsule or cell wall. We have shown that despite its ability to activate complement through C3 as well as anticapsular antibody, anti cell wall IgG is deficient at mediating a) *in vivo* clearance; b) *in vitro* phagocytosis, and c) immune adherence (Brown, Hosea, Hammer, Burch, Frank).

Finally, in order to pursue our interest in the effects of C3b deposited on the cell surface, that bovine conglutinin binds very specifically to a hemolytically inactive form of cell surface C3b, C3bi, and that this fact may be used as the basis for a quantitative assay of C3bi (Brown, Gaither, Hammer, Hosea, Frank).

The mechanism of resistance of gram negative bacteria to killing by fresh serum alone has been studied. We have measured uptake on bacteria as well as consumption from the fluid phase of terminal complement components (C5-C9) by a variety of bacterial types (Joiner, Brown, Hammer, Frank).

The uptake and binding of ^{125}I labelled C3, C5, C7 and C9 was measured on rough, serum-sensitive and smooth, serum-resistant strains of Salmonella minnesota. Consumption of individual complement components from serum has been correlated with uptake of the same components on the bacterial surface. The molecular form and binding characteristics of the terminal components on the surface of the serum-sensitive and serum-resistant bacteria was assessed using sucrose density gradient ultracentrifugation and SDS-PAGE analysis of labelled components eluted from the organisms. Elution of terminal components from the bacterial surface by salt, detergents and proteases was also measured.

Terminal complement components bind in substantial amounts to the surface of both serum sensitive and serum resistant S. minnesota. Moreover, C5, C7 and C9 are completely consumed in the reaction of the serum-resistant organism with serum. Unlike serum sensitive bacteria, however, the binding of C5, C7 and C9 to serum-resistant bacteria is not firm and is mediated primarily by ionic, rather than hydrophobic interactions. When the terminal attack complex consisting of C5-9 was completed but not before, the C5-9 complex was released from the bacterial surface. In summary, a membrane attack complex (MAC) forms on the surface of a smooth, serum-resistant strain of S. minnesota, but the MAC is not bactericidal because it fails to insert into the hydrophobic region of the bacterial outer membrane (Joiner, Brown, Hammer, Frank).

Significance to Biomedical Research:

Our laboratory is one of the few in the country attempting to determine how the interaction of antibody and complement lead to the production of specific diseases as well as influence the host defense process. To this end we are making major efforts to determine how the specific cleavage fragments formed on complement activation act in biological systems. Ultimately, we wish to know how these fragments contribute to the development of disease. At present we are focused on the opsonic fragments of complement which are responsible for the phagocytosis of bacteria and protection from infection. A wide variety of projects have examined the interaction of these fragments with specific receptors, the way the body handles the biologically active fragments when they are formed, and the results of the interaction of the biologically active fragments with those receptors. This has led to a wide variety of findings, both in biochemical terms and in practical terms in the case of our understanding of the mechanisms of bacterial resistence.

Most assays of response to the pneumococcal vaccine do not measure IgG antibody specifically which our studies suggest is the most important antibody class. In addition, patients at high risk of infection by virtue of their inability to make type specific IgG antibody would potentially benefit from gamma globulin replacement therapy.

Publications:

1. Hammer, C.H., Wirtz, G.H., Renfer, L., Gresham, H.D. and Tack, B.F.: Large scale isolation of functionally active components of the human complement system. J. Biol. Chem. 256: 3995-4006, 1981.

2. Tack, B.F., Janatova, T., Harrison, R.A. and Hammer, C.H.: The third, fourth and fifth components of human complement: Isolation and biochemical properties. In L. Lorand (Ed.): Methods in Enzymology, Part C, Proteolytic Enzymes, 1981. In press.

3. Hansch, G.M., Hammer, C.H., Mayer, M.M., and Shin, M.L.: Activation of the fifth and sixth component of the complement system: Similarities between C5b,6 and $C(5b)^{a}$ with respect to lytic enhancement by cell-bound C3b or A2C, and species preferences of target cells. J. Immunol., 1981. In press.

4. Hansch, G.M., Hammer, C.H., Vanguri, P., and Shin, M.L.: Self versus non-self species restriction in the lysis of erythrocytes by the terminal complement proteins. Proc. Natl. Acad. Scie. USA, 1981. In press.

5. Hansch, G.M., Hammer, C.H., Jiji, R., Rother, U., and Shin, M.: Lysis of paroxysmal nocturnal hemoglobinuria erythrocytes by acid-activated serum. Blood, submitted for publication.

6. Berger, M., Gaither, T.A., Hammer, C.H. and Frank, M.M.: Lack of binding of human C3, in its native state, to C3b receptors. J. Immunol., 127: In press, 1981.

7. Brown, E.J., Hosea, S.W. and Frank, M.M.: Reticuloendothelial clearance of radiolabelled pneumococci in experimental bacteremia: correlation of changes in clearance rates, sequestration patterns and opsonization requirements at different phases of the bacterial growth cycle. J. Reticuloendoth. 36: 25-31, 1981.

8. Hosea, S.W., Brown, E.J. and Frank, M.M.: The critical role of complement in experimental bacteremia, J. Infect. Dis., 142: 903-909, 1980.

9. Brown, E.J., Hosea, S.W. and Frank, M.M.: The role of complement in the localization of pneumococci in the splanchnic reticuloendothelial system during experimental bacteremia, J. Immunol., 126: 2230-35, 1981.

10. Brown, E.J., Hosea, S.W. and Frank, M.M.: The role of the spleen in experimental pneumococcal bacteremia, J. Clin. Invest., 67: 975-82, 1981.

11. Brown, E.J. and Frank, M.M.: Complement Activation. Immunology Today. In press.

12. Brown, E.J., Hosea, S.W. and Frank, M.M.: Interactions of antibody and complement in experimental pneumococcal bacteremia. I. Anticapsular antibody, submitted for publication.

13. Brown, E.J., Hosea, S.W., Hammer, C.H., Burch, C. and Frank, M.M.: Interactions of antibody and complement in experimental pneumococcal bacteremia. II. Complement Activation and fixation, submitted for publication.

14. Brown, E.J., Gaither, T., Hammer, C.H., Hosea, S.W. and Frank, M.M.: The use of conglutinin in a quantitative assay for the presence of cell bound C3bi, submitted for publication.

15. Magrath, I.T., Freeman, C.B., Santaella, M., Gadek, J., Frank, M., Sprizel, R.J., Novikos, L.: Induction of complement receptor expression in cell lines derived from human undifferentiated lymphomas II Characterization of the induced complement receptor and demonstration of the simultaneous induction of EBV receptor. J. Immunol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00046-13 LCI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Delayed Hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00047-12 LCI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Clinical Studies of Patients with Known or Suspected Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Most work concentrated upon leishmaniasis, schistosomiasis, and filariasis, although a variety of other parasitic infections were seen. Follow-up on the cases with diffuse cutaneous leishmaniasis (DCL) was continued. In contrast to the DCL patients, those with positive leishmanin skin tests in the same endemic area exhibited blastogenic responses to leishmanial antigens. One new case and a drug-treated relapse were treated with local heat at the NIH. Immunologic modulation which depresses both immediate and cell-mediated reactions in chronic schistosomiasis was reversed in patients after treatment of their infections. The development of assays for antibodies to a variety of parasite antigens promises to be useful for both diagnosis and better understanding of pathophysiology of certain parasitic infections. It is now possible to measure parasite-specific IgE to schistosome and filarial antigens, including different stages of the parasites. Also, the proportion of parasite specific IgE and IgG can be estimated. IgE antibodies were found to show greater specificity than IgG to different species of filarial parasites in sera of infected patients. An ELISA test, using cultured larvae as antigen, was developed for serodiagnosis of human strongyloides infection. Results of treatment of *S. mekongi* infections with Praziquantel were excellent with only mild side effects.

Project Description:

This report covers the clinical investigations carried out by the three investigators in the Clinical Parasitology Section (Drs. Neva, Nash and Ottesen). Their experimental or nonclinical research is described in the Annual Report from the Laboratory of Parasitic Diseases. Therefore, there will be some overlap in reporting and in publications listed. The Clinical Parasitology Section provides consultative service in clinical parasitology to the Clinical Center and some members of the Section also participate as infectious disease consultants at the Clinical Center and at the Naval Medical Center. Some of the studies covered in this report were carried out in collaboration with investigators in the Dominican Republic, India and in Puerto Rico.

Pathogenesis and Immunology of leishmaniasis.

Our studies on diffuse cutaneous leishmaniasis (DCL) in collaboration with Dr. Bogaert's group in the Dominican Republic (Dom. Rep.) have continued. Several NIH investigators spent several weeks in the Dom. Rep. searching for additional cases, skin testing individuals in the endemic areas without evidence of leishmaniasis, and studying lymphocyte blastogenesis in positive and negative skin reactors. The DCL cases are limited to a total of only about 20 patients and none are really of the ulcerative variety of cutaneous leishmaniasis. However, positive leishmanial skin test reactors without evidence of past or present cutaneous lesions are not uncommon. Although blastogenic lymphocyte responses in positive skin test reactors are quite variable, their cells are generally reactive, in contast to lack of response in the DCL cases. At 6 months post-treatment the four DCL patients treated at NIH continued to be free of new lesions. But at one year, two of the three who had received amphotericin B had relapsed. The case treated with local heat remained in remission. (Petersen, A. Barral, R. Correa-Coronas, and Neva). One new case of DCL and one of the cases relapsing after amphotericin came to the Clinical Center for treatment with local heat. Immediate response to heat treatment, based on cultures from pre- and post-treatment biopsies as well as clinical appearance of lesions was favorable (Neva, Petersen, and Corsey).

Immune response and treatment of Schistosomiasis.

Previous work on the modulation of immune response in chronic schistosome infections of man has been continued. It is now possible to measure parasite-specific IgE (Hussein and Ottesen). A reduction in levels of schistosome-specific IgG "blocking antibodies" that inhibit antigen induced histamine release from IgE sensitized basophils in the blood of chronically infected patients was found after treatment with oxamniquine. Another result of treatment in these patients was a significant increase in cell-mediated immune responses to parasite antigens, as has been noted before (Hofstetter and Ottesen). Further observations have been made on the antibody response to two carbohydrate antigens from schistosome worms, confirming the usefullness of assays for these antibodies to determine duration and degree of human infections with this parasite (Nash).

Twelve Laotian refugees with *S. mekongi* infection, a newly recognized schistosome similar to *S. japonicum*, were evaluated clinically and treated in a double-blind protocol with the new drug, Praziquantel. Five additional patients, infected with other schistosome species, including two with *S. japonicum*, were also treated with Praziquantel. Results have been excellent with complete cure in all except one case that was lost to follow-up. The drug gave only mild and reversible side effects (Nash, Hofstetter, Ottesen, Barral, and Cheever).

Immune response to filarial and other helminth infections.

Several different assays for parasite-specific IgE in patients with filariasis have been developed. Allergic sensitization to filariae is highest in patients with tropical eosinophilia (Ottesen, Hussain and Anwar). Patients whose disease was manifested by elephantiasis and recurrent episodes of lymphangitis were found to have high levels of circulating immune complexes (Ottesen, Lawley, and Lunde). IgE antibodies were found to show a greater degree of species specificity than IgG to several different species of filarial parasites in sera of infected humans (Weiss, Hussain, and Ottesen). An ELISA test, using larvae of *Strongyloides stercoralis* and *S. ratti* as antigens, was developed and found to detect antibodies in 80 to 85% of parasitologically proven infections. Cross-reacting antibodies to *Strongyloides* antigens in patients with other helminthic infections are not common so this test should be clinically useful (Neva).

Publications:

1. Hofstetter, M., Nash, T. E., Cheever, A. W., dos Santos, J. G., and Ottesen, E.A.: Schistosomiasis mekongi in Southeast Asian refugees. J. Inf. Dis. (in press).

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8. Ottesen, E.A.: Tropical Eosinophilia. In Wyngarden, J.B. and Smith, L.H. (Eds): Cecil Textbook of Medicine, 16th edition. W.B. Saunders Co. (in press)

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 A1 00048-11 LCI

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Pathophysiology of Autoimmune Hemolytic Anemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immunology Section and Chief, LCI	LCI	NIAID
OTHER:	L. Fries	Clinical Associate	LCI	NIAID
	S. Inada	Visiting Fellow	LCI	NIAID

COOPERATING UNITS (if any)

T. Lawley, R. Hall, S. Katz (Dermatology Branch, NCI/NIH); G. Crabtree (NCI/NIH); H. Moutsopoulos (NIDR/NIH); and T. Chused (NIAID)

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Clinical Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 5/12	1 5/12	0

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tests have been developed for the study of reticuloendothelial system function in man and have been widely applied to patients with autoimmune disease. Moreover, it has been found that defects in reticuloendothelial system function exists with patients with particular HLA types, namely HLA B8 DRw3, known to be associated with the development of autoimmune disease raising important questions as to the pathogenesis of the syndrome. Tests have now been developed to ennumerate Fc receptor number on peripheral blood monocytes as well as average association constant of those Fc receptors. These tests are being applied to autoimmune disease patients as well as patients with a variety of other illnesses.

Project Description:

Objectives:

To define the pathophysiologic function of antibody and complement on the surface of red cells in patients with hemolytic anemia, and to use the findings of this study to develop new tests for immunologically specific, reticuloendothelial system macrophage membrane receptors in patients with a variety of disease states. The tests developed for the study of autoimmune hemolytic anemia have been found to have wide application to other autoimmune and immunologic problems.

Methods Employed:

These studies examine the clearance of radiochromated erythrocytes from the circulation. The initial studies were performed in a guinea pig model. The recent studies have been performed in human volunteers or in patients with various diseases. In the initial studies in humans, the clearance of erythrocytes coated with IgM isoagglutinin antibodies with or lacking membrane bound complement was examined. In later studies, radiochromated erythrocytes were coated with highly purified human IgG anti-Rh antibodies and the survival of these cells was also studied. The third general set of clearance studies examined the clearance of radioactive aggregated albumin. This is the standard method for determining RES clearance and can be used to determine both liver blood flow and the ability of the reticuloendothelial system to clear particulate materials.

Major Findings:

In past years, we identified the immunologically active protein fragments which, when deposited on red cells, are responsible for the clearance of those cells from the circulation. It was reported that receptors for these immunologically specific fragments were present on the membrane of macrophages and the receptors were responsible for removal of cells coated with these fragments from the circulation. It became clear that it was possible to study patients with a wide variety of diseases to determine whether their receptors for these immunologically active fragments were functioning normally *in vivo*. It has been known for years that the reticuloendothelial system (RES) plays a major role in removing foreign material from the circulation. These foreign materials include bacteria and parasites, as well as endogenously generated immunoreactive materials such as immune complexes. It was suspected that defects in RES function might be important in both infectious and immunologic diseases. However, when this question was examined directly using the techniques available, no defects in RES function were noted in any disease state in which blood flow to the RES organs was normal. Until the present studies, the technique available for examination of RES function was that of study of clearance of aggregated albumin from the circulation. This study has led to the development of new techniques for evaluation of RES function. Using these new techniques developed within our unit, we have shown that there are indeed diseases with defects in RES function. Thus, receptors for complement and immunoglobulin on a patient's macrophages will recognize these immunologically active materials coating foreign substances as they circulate.

The defects are quite specific for different sets of receptors. Our test utilizes the patient's own red blood cells. The cells are coated with antibody and/or complement fragments and are infused into the patient and their clearance is followed. Using this technique, we have found that almost every patient with active lupus erythematosus has a major defect in Fc receptor specific clearance, although the clearance of aggregated materials is normal. We find that as the disease improves, the clearance defect also tends to improve and the complexes present in the circulation tend to disappear. However, it is striking that most patients with lupus erythematosus have a persistence of their clearance defect for long periods of time, even on treatment.

Studies of Sjögren's Syndrome demonstrated that patients with clearance defects and circulating immune complexes tend to develop peripheral manifestations of their Sjögren's Syndrome and extensive tissue injury. On the other hand, patients who do not have clearance defects tend not to develop secondary manifestations of their Sjögren's Syndrome with peripheral tissue destruction, even in the presence of circulating immune complexes.

We studied patients with Dermatitis Herpetiformis in collaboration with Drs. Lawley, Hall and Katz of the Dermatology Branch, NCI. It was found that 50% of these patients have a clearance defect. Because these patients have a particular HLA type, B8 DW 27, we studied normal volunteers with this haplotype. 50% of these normal individuals had an Fc receptor defect. Moreover these patients were shown by Dr. Fauci to have a defect in T lymphocyte Fc receptor expression. Thus, the defect in Fc receptors may precede the development of disease. Thus study provides the first link between HLA type and autoimmune disease and may prove a critical link between the known association of certain HLA types and autoimmunity.

Because of these findings it became essential to determine the number, as well as the affinity, of Fc receptors on cells in patients with autoimmune disease and in patients with various known HLA types. Two methods were developed to study this. In the first simpler technique a rosette assay was developed to examine the binding of IgG coated cells to peripheral blood monocytes after they had adhered to glass plates. This is a standard method of examining Fc receptor activity. Detailed analysis of the peripheral blood monocytes of normal individuals with the HLA B8 haplotype as compared with normals has demonstrated no difference in the number of Fc receptors by this technique (Inada and Frank). Because this technique is essentially indirect it was necessary to develop a direct technique to more carefully examine this same issue. A specific binding assay of monoclonal IgG subclass 1 myeloma protein to the Fc receptor on cells was developed (Fries, Crabtree, Frank). This binding has allowed us to determine the number of Fc receptors on circulating peripheral blood monocytes in patients as well as in normal individuals to determine the specific number of receptors as well as the average association constant. This technique has also confirmed the original observations of Inada, showing no difference in number of Fc receptors in this case on the circulating peripheral blood cells. This powerful new technique is allowing us to examine a variety of autoimmune diseases to determine the number and average association constant of the Fc receptors. This material is currently being studied.

Significance to Biomedical Research:

These techniques allow for the complete reevaluation of reticuloendothelial system function in man. Already they are being widely applied. The use of these techniques has already allowed for the description of the first patients with receptor specific defects. C3b specific defects have been demonstrated in primary biliary cirrhosis and Fc receptor defects in a number of serious autoimmune diseases. These techniques provide a powerful new tool in the evaluation of these patient groups and provide a new approach to understanding the pathophysiologic basis of these diseases.

Proposed Course:

We intend to extend these studies to additional autoimmune diseases. First, we hope to determine how RES specific defects contribute to the development of autoimmune disease. Second, we hope to determine the nature of the underlying defect which leads to the RES abnormality. Clearly this is not in the number of circulating Fc receptors on peripheral blood monocytes. Third, we intend to widen this technique to understanding how these specific receptor-like and interactions lead to the host defense process. Studies in that area are included in project AI 00045-13 LCI in this report.

Publications:

1. Lawley, T.J., Hall, R.P., Fauci, A.S., Katz, S.I., Hamburger, M.I., and Frank, M.M.: Defective Fc receptor functions associated with the HLA-B8/DRw3 Haplotype: Studies in patients with dermatitis herpetiformis and normal subjects. N. Eng. J. Med. 304: 185-192, 1981.

2. Frank, M.M.: The function of the reticuloendothelial system in autoimmune diseases. In N. Cummings (Ed.): Immune Mechanisms in Renal Disease. in press.

3. Hamburger, M.I., Lawley, T.J., Kimberly, R.P., Plotz, P.H. and Frank, M.M.: A serial study of reticuloendothelial system Fc receptor function in patients with systemic lupus erythematosus, submitted for publication.

4. Hamburger, M.I., Lawley, T.J., Hall, R.P., Moutsopoulos, H.M., Sharp, G.C., and Frank, M.M.: Reticuloendothelial system function in mixed connective tissue disease, submitted for publication.

5. Frank, M.M.: In Vivo studies of Fc receptor dependent clearance: Defects in Autoimmune Disaese and Associated with the HLA B8 DRw3 Haplotype. In Proceedings of the VIII International Immunopathology Symposium. Submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00050-11 LCI

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Clinical Studies of Complement Abnormalities of Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immunology Section and Chief, LCI	LCI	NIAID
OTHER:	S. Hosea	Senior Investigator	LCI	NIAID
	K. Katusha	Microbiologist	LCI	NIAID
	C. Brickman	Clinical Associate	LCI	NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Clinical Immunology

INSTITUTE AND LOCATION

NIH/NIAID, Bethesda, MD 20205

TOTAL MANYEARS: 1 11/12	PROFESSIONAL: 11/12	OTHER: 1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The general objective of this program has been to define the role of complement in human illness and to better develop methods of therapy for immune damage mediated by complement. Major emphasis was placed on studies of patients with hereditary angioedema. Because the detailed projects of this study have extensively overlapped with the two earlier projects, Nos. Z01 AI 00045-13 and 00048-11, it has been decided to eliminate this project and include the findings in those other projects following the presentation this year.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00057-08 LCI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Basic Studies on Pathogenic Fungi

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.J. Kwon-Chung	Senior Investigator	LCI	NIAID
OTHER:	Itzhack Polacheck	Visiting Fellow	"	"
	Judith C. Rhodes	Postdoctoral Fellow	"	"
	J.E. Bennett	Head, Clinical Mycology Section	"	"

COOPERATING UNITS (if any)
A.K. Bhattacharjee (NIAMDD)
V.J. Hearing (NCI)
S.E. Straus (LCI, NIAID)

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5	3	2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interests and proposed course of our unit are to cover basic and applied aspects of various pathogenic fungi including their morphology, taxonomy, pathology, epidemiology, biochemistry and genetics. Topics of present interest include: 1) structure of the capsular polysaccharide of serotype A Cryptococcus neoformans, 2) developing a new improved medium for the identification of serotype A-D and B-C group of C. neoformans, 3) phenoloxidase as one of the virulence factors in C. neoformans and 4) a new special staining method for histopathological diagnosis of cryptococcosis.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

Objectives of the project cover both basic and applied aspects of various pathogenic fungi including their morphology, taxonomy, pathogenicity, life cycle, biochemistry and genetics. The topics of present interest include: 1) chemical structure of capsular polysaccharide of serotype A of *Cryptococcus neoformans*, 2) phenoloxidase as one of the virulence factors of *C. neoformans*, 3) genetic relatedness of *C. neoformans* and *C. bacillisporus*.

Methods Employed:

Production of Capsular Polysaccharide: Cultures were grown on Sabouraud dextrose broth, and cells were killed by adding buffered formalin and removed by centrifugation. The supernatant was treated with sodium acetate, acetic acid and 95% ethanol to precipitate polysaccharide. The polysaccharide was dissolved in distilled water and treated with sodium acetate and acetic acid. The solution was deproteinated with chloroform and butanol. The precipitate was redissolved in distilled water and reprecipitated with 95% ethanol. The polysaccharide was chromatographed on a column of DEAE-cellulose using 0.01 M phosphate buffer, pH 7.3, and a linear salt gradient of 0-1 M NaCl. The 80% of material was eluded at a salt conc. 0.2 M, and this material was dialized and freeze dried.

Chemical Structure of the Polysaccharide: Gel filtration, ultracentrifugation, paper chromatography, hydrolysis by 1 N HCl, methylation, periodate oxidation and chromium trioxide oxidation methods were used to anlayze the chemical structure of the polysaccharide.

Examination of C. neoformans var. gattii and C. bacillisporus: The type cultures of *C. neoformans* var. *gattii* and *C. bacillisporus* were compared for their temperature tolerance, ℓ-malate assimilation, creatinine-glucose-bromothynol blue reaction, mouse pathogenicity and the ability to cross with *Filobasidiella neoformans* and *F. bacillispora*.

Phenoloxidase Assays: O-diphenoloxidase of *C. neoformans* was assayed by a spectrophotometric method as well as a radiometric method. In the spectrophotometric assay, dopachrome and 5, 6 hydroxy inodole were measured at 480 and 300 nm, respectively, after the extracted enzyme of *C. neoformans* was incubated with L-Dopa or norepinephrine for 30 min. For the radiometric assay, C^{14} labeled L-Dopa and norepinephrine were used as the substrates, and the radioactive melanine resulting from the enzyme-substrate reaction was measured according to the Hearing, *et al.* method.

Localization of Phenoloxidase in C. neoformans Cells: To detect the membrane-bound phenoloxidase, protoplasts were produced by incubating *C. neoformans* with β-glucuronidase. The protoplasts were then incubated with glutaraldehyde before rupturing them. The enzyme activity was measured by a radiometric assay.

Isolation of the Isogenic Sets of Phenoloxidase Lacking Mutant: The *a* mating type tester strain of *C. neoformans* was streaked on L-Dopa agar medium repeatedly to select albino or only lightly pigmented colonies. A beige-colored colony was isolated and crossed with α type of tester strain. The F_1 progeny were screened on L-Dopa agar medium. Three each of white (mel$^-$) and dark (mel$^+$) progeny were selected to test their virulence for mice. The mel$^-$ isolates were repeatedly streaked on Dopa agar to isolate revertant (mel^R) colonies. The isogenic sets of mel$^-$ and mel^R isolates were compared for their virulence.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) Significant Findings in the Area of Polysaccharide Chemistry: An isolate of *C. neoformans* which was originally serotyped as A yielded two kinds of clones: serotype A and serotype D. The chemical structure of the capsular polysaccharide from the serotype A isolate showed that all of the D-mannosyl residues in the main chain carry a single substituent and no unsubstituted D-mannosyl residues present. In contrast, the characteristic feature of serotype D polysaccharide is that it contains a substantial proportion of unsubstituted D-mannosyl residues in the main chain.

2) Findings in the Area of Biochemistry: Phenoloxidase of *C. neoformans* is very different from human and mushroom phenoloxidase in that tyrosine can not be utilized as the substrate for melanin production in *C. neoformans*. The phenoloxidase of *C. neoformans* is similar to the phenoloxidase from *Mycobacterium leprae* but different in the inhibitors. Mimosine acts as an inhibitor for the enzyme in *C. neoformans* but serves as a substrate for melanin synthesis in *M. leprae*. The phenoloxidase of *C. neoformans* is a constitutive and membrane-bound enzyme which can use only di or polyphenolic compound but not monophenolic compound. The two intermediates of the melanin synthesis in *C. neoformans* were identified and they are dopachrome and 5, 6 hydroxyindole.

3) Findings in the Area of Genetics: The DNAs of serotypes A and D of *C. neoformans* demonstrated relatedness value of 88 to 94%. DNAa of serotypes B and C isolates showed 89% relatedness. Hybridizations of DNAs of A or D with those of B or C, however, yielded relatedness values of only 55 to 63%, indicating that these DNAs are significantly different.

4) Findings in the Area of Virulence: The virulence tests with mel$^-$ and mel^R isogenic sets showed that the isolates lacking phenoloxidase are avirulent for mice. The revertants which recovered the enzyme became highly virulent for mice. The mel$^-$ isolates which lost the enzyme but had intact uptake system for Dopa grew poorly in mouse brain while mel^R grew extensively. The mel^R produced hydrocephalus within 2 weeks by the inoculum of 1×10^4 cells (i.v.). The mel$^-$ mutants which lacked phenoloxidase and lost the active transport system for Dopa or norepinephrine completely failed to multiply in the mouse brain. These results indicate that the phenoloxidase of *C. neoformans* plays an important role for the mouse virulence.

Proposed Course:

The studies on virulence factors of *C. neoformans* will be continued. The nitrogen metabolism of two serogroups of *C. neoformans* will be studied.

Publications:

1. Bhattacharjee, A.K., Kwon-Chung, K.J. and Glaudemans, C.P.J.: Structural studies on the major capsular polysaccharide from *Cryptococcus bacillisporus* serotype B. *Carbohydrate Research* 82:103-111, 1980.

2. Rogers, A.L., Kwon-Chung, K.J. and Flegler, S.L.: A scanning electron microscope comparison of basidial structures in *Filobasidiella neoformans* and *F. bacillispora*. *Sabouraudia* 18:85-89, 1980.

3. Kwon-Chung, K.J. and Hill, W.B.: Sexuality and pathogenicity of *Filobasidiella neoformans* (*Cryptococcus neoformans*). In Vanbreuseghem, R. and DeVroey, C. (Eds.): *Sexuality and Pathogenicity of Fungi*. New York, N.Y. Masson, 1981, pp. 243-250.

4. Kwon-Chung, K.J. and Hill, W.B.: Virulence of the two mating types of *Emmonsiella capsulata* and the mating experiments with *Emmonsiella capsulata* and *E. capsulata* var. *duboisii*. In Vanbreuseghem, R. and DeVroey, C. (Eds.): *Sexuality and Pathogenicity of Fungi*. New York, N.Y., Masson, 1981, pp. 48-56.

5. Aulakh, H.S., Straus, S.E. and Kwon-Chung, K.J.: Genetic relatedness of *Filobasidiella neoformans* (*Cryptococcus neoformans*) and *Filobasidiella bacillispora* (*Cryptococcus bacillisporus*). *Int. J. Syst. Bacteriol.* 31:97-103, 1981.

6. Rhodes, J.C., Kwon-Chung, K.J. and Popkin, T.J.: Ultrastructure of the septal complex in hyphae of *Cryptococcus laurentii*. *J. Bacteriol.* 145: 1410-1412, 1981.

7. Kwon-Chung, K.J., Hill, W.B. and Bennett, J.E.: New, special stain for histopathological diagnosis of cryptococcosis. *J. Clin. Microbiol.* 13: 383-387, 1981.

8. Bhattacharjee, A.K., Kwon-Chung, K.J. and Glaudemans, C.P.J.: Capsular polysaccharides from a parent strain and a putative, mutant strain of *Cryptococcus neoformans* serotype A. *Carbohydrate Research*. In press.

9. Khan, S.R., Kimbrough, J.W. and Kwon-Chung, K.J.: Ultrastructure of *Filobasidiella arachnophila*. *Can. J. Bot.* In Press.

10. Kwon-Chung, K.J., Bennett, J.E. and Rhodes, J.C.: *Cryptococcus neoformans* var. *gattii* and its synonym *Cryptococcus bacillisporus*. *Ant. van Leeuwenhock*. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00058-07 LCI

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The pathogenesis and chemotherapy of herpes virus infections in man.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. E. Straus	Senior Investigator, LCI, NIAID
Other:	H. Takiff	Clinical Associate, LCI, NIAID
	H. S. Aulakh	Fogarty International Fellow, LCI, NIAID (until Dec., 1980)

COOPERATING UNITS (if any) J. Costa (LP, NCI), G. Vande Woude (LMV, NCI), J. Hay (USUHS), W. Ruyechan (USUHS), S. Houff, and W. Wallen (LID, NINCDS), and R. Whitley (Birmingham, Alabama).

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Medical Virology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	1.5	1.5

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The pathogenesis, molecular biology, immunology, natural history, and therapy of human herpes virus infections are being investigated. Immunocompetent and immunodeficient patients with a wide range of herpes virus infections have been identified, followed clinically, diagnosed by virus isolation, studied immunologically, and, in some cases, treated successfully with systemic antiviral chemotherapy.

Our major focus on the study of molecular biology and latency of varicella zoster virus DNA has continued. During the past year we have completed studies on the restriction endonuclease analysis and molecular epidemiology of varicella zoster virus DNA. We have molecularly cloned a library of varicella zoster DNA fragments, and have nearly completed physical mapping of varicella zoster virus genome.

Project Description:

Objectives:

1) To determine the pathogenesis and natural history of herpes virus infections in both normal and immune compromised humans.

2) To evaluate the efficacy of antiviral agents in the treatment of human herpes virus infection. These infections include both minor and serious diseases produced by herpes simplex types 1 and 2, varicella-zoster virus, cytomegalovirus, and EB virus.

Methods Employed:

1) Patients with suspected herpes virus infections are referred to the Medical Virology Section from the Clinical Center, the National Naval Medical Center, local hospitals and practicing physicians. The patients are evaluated and appropriate specimens are collected for virus isolation and serologic testing.

2) Virus isolation is performed in primary or continuous human or simian cell lines. The viruses are identified by the appearance of typical cytopathic changes and typed by immunofluorescent procedures where required.

3) In selected cases, the viral DNA has been purified, digested by restriction endonucleases and analyzed by agarose gel electrophoresis.

4) The clinical efficacy of adenine arabinoside, adenine arabinoside monophosphate, and acyclovir is being investigated in the treatment of herpes virus infections. This unit is collaborating in an NIAID-sponsored multicenter trial evaluating antiviral agents in the treatment of herpes simplex encephalitis, herpes zoster infection of the immune compromised host, and chronic mucocutaneous herpes simplex infections.

Major Findings:

1) During the past year approximately 60 patients have been identified and examined at the NIH with a wide variety of herpes virus infections. Approximately 40 of these patients have had recurrent genital herpes virus infections. Attempts to definitively diagnose the infections by virus isolation have been made in all instances and have been successful most of the time. Over 1,000 virus cultures have been performed with specimens obtained from these patients. In selected individuals numerous specimens have been obtained to quantitiatively assess virus shedding during the course of natural and treated infections and provide material for studies of the biology of the viruses and of their latency.

2) Immunological studies: All patients have been studied in a general way to determine whether lymphocyts, immunoglobulin, or complement deficiencies are present. A number of patients with severe herpes virus infections have been studied more exhaustively. A group of five individuals who are suspected of having chronic infectious mononucleosis have now been serially studied and followed. These individuals have been found to have exceedingly high antibody levels to certain EB virus components and one or more subtle abnormalities of T cell or natural killer cell responsiveness to EB virus in vitro.

3) Clinical trials of anti-viral agents:

a) Herpes simplex encephalitis - the data from the initial collaborative trial of Adenine Arabinoside for herpes simplex encephalitis has been published and clearly substantiate the previously published preliminary findings. A new study has been initiated this year to compare the efficacy of Adenine Arabinoside monophosphate or acyclovir with Adenine Arabinoside in the treatment of herpes simplex encephalitis.

b) Varicella-zoster virus infections: The NIAID collaborative study of Adenine Arabinoside for the treatment of varicella-zoster virus infections in a compromised host has been completed and the data is being prepared for publication. Several immune compromised patients with disseminated zoster infection and one seemingly normal individual with severe varicella pneumonia were treated with acyclovir.

c) Mucocutaneous herpes simplex infection: Immunodeficient patients with chronic mucocutaneous herpes simplex infections have continued to be treated under the collaborative Adenine Arabinoside study until recently. This study has now terminated, and the data are being collected for publication. Two immune compromised individuals with severe recurrent genital herpes have been treated successfully one or more times with acyclovir. One individual has been treated successfully through 14 sequential recurrences of severe genital herpes.

d) Cytomegalovirus infection: One immunodeficinet patient with culture proven disseminated cytomegalovirus infection and cytomegalovirus retinitis responded to acyclovir therapy with transient clearing of cultivable virus and stabilization of vision.

4) Molecular biology and molecular epidemiology of varicella-zoster virus DNAs: Varicella-zoster virus specimens have been recovered directly from vesicle fluid specimens of patients with varicella and with zoster infection. The viral DNA purified from these samples has been characterized by a variety of molecular and electromicroscopic techniques. We have completed our analyses of the general genomic organization of the varicella-zoster DNA. In the past months we have molecularly cloned

varicella-zoster virus DNA restriction fragments in a lamdaphage vector. We have characterized a large number of individual clones from which we have selected a library representative of nearly all regions of the viral genome. Blot hybridization experiments have helped us order these fragments into a physical map for varicella-zoster DNA.

We have compared the restriction endonuclease cleavage patterns of varicella-zoster virus DNA eight individual patients. We have been successfully able to demonstrate that the viral DNA of isolates recovered from epidemiologically unrelated individuals possess unique restriction patterns. We have also been able to demonstrate in some individuals that the strain of zoster represents a reactivation of latent virus rather than exogenous reinfections.

Significance to Biomedical Research and the Program of the Institute:

Herpes viruses produce substantial morbidity and mortality each year. Normal adults and children suffer individual or recurrent infections caused by members of the herpes virus family. These infections ragne from rather benign afflications to those such as herpes simplex encephalitis which when untreated causes death in 60-70% of individuals. Immune compromised individuals, of which there are an ever increasing number and variety, are especially susceptivle to herpes infections. The above studies have expanded current knowledge of the natural history, pathogenesis, and biology of these infections. The therapeutic trials in which we collaborate have been the first to clearly demonstrate efficacy of antiviral compounds for these types of infections. These results have been encouraging and pave the way for future studies with even more potent antiviral drugs.

Proposed Course:

During the next year we will continue to see patients with debilitating or life-threatening herpes virus infections. We will continue to search for immune abnormalities which underly some of these infections. Trials involving a number of antiviral drugs will be continued in efforts to control the infections.

We will attempt to complete our molecular cloning of varicella-zoster virus DNA fragments and mapping of the viral genome. We will hopefully begin to utilize these cloned reagents as probes in studies of the latency of varicella-zoster virus in human tissue.

Publications:

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2. Whitley, R.J., Soong, S.J., Hirsch, M.S., Karchmer, A.W., Dolman, R., Galasso, G., Dunnick, J.K., Alford, C.A., and the NIAID collaborative study group. Herpes simplex encephalitis: Vidaribine therapy and diagnostic problems. N. Eng. J. Med. vol. 304, pp. 313-318, 1981.

3. Straus, S.E., Aulakh, H.S., Ruyechan, W.T., Hay, J., Vande Woude, G.F., Owens, J., and Smith, H.A.: Studies of the structure of varicella-zoster virus DNA. Abstract presented to the Herpes Virus Workshop, Cold Spring Harbor, N.Y., August, 1980.

4. Straus, S.E., Owens, J., Takiff, H.E., Vande Woude, G.F., Aulakh, H.S., and Hay, J.: The characterization and molecular cloning of varicella-zoster virus DNA. Abstract presented at the International Workshop on Herpes Viruses, Bologna, Italy. July, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 A1 00154 06 LCI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunologic neurophysiologic, biochemical, and cellular events in immediate hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	Michael A. Kaliner	Senior Investigator	LCI, NIAID
Other	J. Shelhamer	Research Associate	LCI, NIAID
	Z. Marom	Research Associate	LCI, NIAID
	R. Lemanske	Research Associate	LCI, NIAID
	S. Bowman	Clinical Associate	LCI, NIAID
	L. Steel	Staff Fellow	LCI, NIAID
	H. Oertel	Visiting Fellow	LCI, NIAID
	M. Donlon	Guest Worker	LCI, NIAID
	S. Wescott	Lab Technician (biol.)	LCI, NIAID
	J. Dyer	Chemist	LCI, NIAID

COOPERATING UNITS (if any)

Walter Reed Army Medical Center (R. Evans, R. Summers, L. Smith, R. Sigler)

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Allergic Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: PROFESSIONAL: OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Employing quantitation of radiolabeled mucous glycoprotein secretion from cultured human airways, the effects of HETEs, SRS-A (leukotrienes C and D), leukotriene B, and steroids were examined. The synthesis of HETEs by human airways was verified. The effects of prostaglandin generating factor of anaphylaxis (PGF-A) on prostaglandin synthesis by human airways (peripheral and central), pulmonary arteries and veins, and peripheral lung tissue were examined. The roles which the eosinophil and neutrophil play in the generation of late-phase reactions was studied. The histamine receptor subtype on mast cells, as well as the beta adrenergic receptor in both mast cell membranes and perigranular membranes, were characterized. The response of humans to histamine infusions, both in regards to physiologic responses and the receptor subtypes involved, were characterized. The quantitative relationship between autoantibodies to beta adrenergic receptors and autonomic nervous system responsiveness was determined. The assay for histamine in plasma and urine has been made more sensitive. Employing the urine histamine assay, the pathogenesis of reactions to radiocontrast media are being explored. The presence of opiate receptors on rat and human mast cells was examined.

PHS-6040
(Rev. 2-81)

Project Description

Objectives

The goals of the Allergic Diseases Section are to expand our knowledge of all aspects of immediate hypersensitivity reactions. Our particular expertise and focus are defining the biochemical events which accompany and control the triggering of allergic reactions, the neurophysiologic mechanisms which potentially modulate these events, the characterization of the mediators of anaphylaxis, and identification of the responses of relevant target issues to these mediators.

We are concentrating on three primary areas: 1) employing human lung tissue, we study "asthma in a test tube" in order to understand the immunologic triggers, biochemical concomitants and neurophysiologic control of mediator release; the identification of additional mediators of anaphylaxis; and the study of mucous secretion, 2) rat peritoneal mast cells are utilized as a pure source of effector cells which may be readily studied *in vitro* and may amplify as well as initiate new areas of interest in relationship to the human lung model, and 3) clinical allergic asthma is being evaluated in an extensive protocol designed to expand our understanding of the neurophysiologic mileau of allergic individuals as compared with cystic fibrosis and normal subjects.

Methods Employed

1. Human lung model: Lungs removed, usually for cancer resection, almost always contain large amounts of normal, functioning lung tissue. We obtain these lungs in cooperation with the surgical and pathology departments of all the major hospitals in the Bethesda area. The lung tissue is washed, fragmented, and replicated into 200 mg samples and subsequently incubated in dilutions of allergic serum for two hours at 37°C or for eighteen hours at 25°C. After this "sensitization" period, the serum is removed and the fragments challenged with allergen. The interaction of the allergen with tissue (mast cell) bound IgE induces the release of histamine, slow-reacting substance of anaphylaxis (SRS-A), eosinophil chemotactic factor of anaphylaxis (ECF-A), and prostaglandin E_1 and $F_{2\alpha}$ (PGE_1 and $PGF_{2\alpha}$). These mediators may be assayed on the isolated terminal portion of the guinea pig's ileum (histamine, SRS-A), chemotactic chambers (ECF-A) or by radioimmunoassay (PGE_1, $PGF_{2\alpha}$)

Mucous glycoproteins are monitored by incorporating ^{3}H-glucosamine into the mucous glands during an eighteen-hour incubation period. Secretions are precipitated by 95% alcohol and filtered on metricel membranes.

PGF-A is prepared by reversed anaphylaxis of human peripheral lung tissue employing 1:20 to 1:100 dilutions of rabbit anti-human IgE. The supernatant is filtered through DM5 and UM05 membranes and the 500-5000 molecular weight fraction employed to generate PGs and thromboxane.

2. Rat mast cells: rat peritoneal and pleural mast cells are obtained from freshly sacrificed male Sprague-Dawley rats by lavage of these cavities. The peritoneal cells include 5% mast cells (or approximately 500,000 to 1 million/ animal). The cells are used either in mixed cell suspensions or after purification by centrifugation into albumin cushions. Mast cell granules are isolated with intact perigranular membranes by sonication and centrifugation through sucrose cushions. Granules free of membranes are obtained by osmotic lysis of mast cells.

Rat LPR are generated by intradermal injections of rabbit anti-IgE, mast cell granules, or inflammatory factor of anaphylaxis (IF-A). IF-A is isolated from mast cell granules by membrane filtration, gel filtration, and ion-exchange chromatography. Elicitation of LPR is quantitated histologically. Rat eosinophilia was induced by injecting 3000 Sephadex G-200 beads IV producing 600-1000 eosinophils mm^3 in 6 days. Neutropenia in rats was elicited by injections of vinblastine 0.75-1.5 mg/kg IV, with selective neutropenia developing in 4-6 days.

Histamine receptors are quantitated by ^{3}H-pyrilamine binding at 4°C in Hank's Balanced Salt Solution for thirty minutes followed by filtration through silicone oil. Specificity is defined by receptor binding in the presence of unlabeled pyrilamine or histamine. Analyses involve Scatchard plots as well as determination of the concentration of antagonist producing 50% displacement.

Beta adrenergic receptor analyses involved both intact mast cells and mast cell granules isolated with intact perigranular membranes by a combination of controlled sonication and sucrose density gradients. Ligand binding employed dihydroalprenolol and involved membrane filtration.

3. Clinical asthma study: the methods employed in this study are covered in detail in the clinical protocol, and include: a) measurement of cutaneous blood flow by ^{133}xenon disappearance, b) pupillary responses as measured by a binocular pupillometer, c) airway obstruction as measured by flow-volume curves with and without helium inhalation, and d) serum cyclic nucleotide responses as measured after intravenous isoproterenol administration.

An additional study completed this year involved examining the effects of histamine infused into normal subjects in comparison to mild allergic asthmatics. Techniques employed consisted of monitoring pulse rate, blood pressure, peak expiratory flow, skin test responses, skin temperature, urinary histamine, plasma histamine, plasma cyclic nucleotides, and plasma prostaglandins.

Major Findings

1. Human lung studies:

   a. Mucous secretion

   Mixtures of synthetic monohydroxyeicosatetraenoic acids (HETEs) increased mucous secretion at micromolar concentrations. We therefore studied purified 5-, 8-, 11-, 12-, and 15-HETEs and found them active at 10-100 nM. Supernatants from airway cultures were analyzed for HETE production by high pressure liquid chromatography and 5-, 8-, 12-, and 15-HETEs were observed at concentrations ranging up to 100-500 nM. Thus, airways produce HETEs, and HETEs are potent secretogogues of mucus.

   Slow-reacting substance of anaphylaxis is derived from 5-HETE and is a mixture of leukotriences LTC$_4$ and LTD$_4$. Employing biosynthesized LTC$_4$ and LTD$_4$, their effects on mucous secretion were determined. Both LTD$_4$ and LTC$_4$ produced dose-related increases in mucous secretion from 10-1000 pg/ml. The SRS-antagonist FPL 55712 reduced the effects of both leukotrienes. LTC$_4$ and LTD$_4$ are the most potent mucous secretagogues currently recognized.

   Corticosteroids (CCS) (dexamethasone and prednisolone) inhibit mucous secretion in a dose-related fashion (10^{-5}-10^{-9}M) with a peak effect (-40%) at sixteen to twenty-four hours incubation. CCS reduce the increases in mucous secretion produced by 5-HETE, histamine, and methacholine but not completely.

Upon close analysis, the effects of CCS upon these secretagogues is to lower baseline mucous secretion but not to influence the increment of increased secretion evoked by secretagogues.

LTB_4 (cis, trans, trans configuration) also increases mucous secretion (10-100 ng/ml), is less potent than LTD_4 or LTC_4 (requiring higher concentrations for activity), but is more potent than histamine or the prostaglandins. LTB_4 (all trans configuration) is inactive.

2. Rat mast cells

a. Late-phase reactions (LPR)

LPR in rats does not produce tissue eosinophilia in contrast to humans in which eosinophilia is a prominent feature. In order to analyze if peripheral eosinophilia might influence the cellular infiltrates in LPR, rats were made eosinophilic (600-1000 eosinophils/mm^3 versus normal of ∿ 100/mm^3). Peripheral eosinophilia failed to modulate the intensity or duration of LPR nor were impressive numbers of eosinophils attracted to the LPR at 1.5, 8, 24, or 72 hours after its onset. However, statistically significant increased eosinophils were found in LPR in eosinophilic rats, and there was a correlation between peripheral eosinophil counts and the eosinophilic infiltrates.

The first phase of LPR is rich in neutrophils. In order to examine if the neutrophils were essential to the development of the second—or mononuclear—phase of LPR, rats were made neutropenic with vinblastine. Selective neutropenia developed (average PMN counts $<$ 50 mm^3) and the animals had LPR elicited with anti-IgE and mast cell granules (MCG). Neutropenic animals failed to develop LPR. Specificity of this reaction was confirmed by sensitizing animals with ovalbumin in complete Freund's adjuvant. These animals developed classical delayed hypersensitivity and were not affected by neutropenia. Thus far, reconstitution of neutrophils employing peritoneal cassein-elicited neutrophils has not been successful.

Therefore, rat LPR is eosinophil-poor even in eosinophilic animals and the later phase of LPR depends upon the elicitation of a neutrophil infiltration early in the course.

b. Beta-adrenergic receptors

Mast cell granule membranes fuse to the limiting membrane during exocytosis. In order to determine if receptors on the granule membrane might replenish or augment cell membrane receptors, β-adrenergic receptors on perigranular membranes were determined. The granule membrane has a single population of β-receptors with K_D = 7.0 nM and B_{max} = 56.6 fmol/ 10^9 granules. In contrast, the cell membrane of rat mast cells has two populations of β-receptors: one with a K_D of 10.6 nM with 186 fmol/10^6 mast cells and a low affinity site with a K_D of 129 nM with B_{max} = 1200 fmol/10^6 cells. Therefore, mast cell granule membranes and cell membranes have similar high affinity receptors with the cell membrane also having a low affinity, high capacity receptor. The role for these receptors is unclear.

c. Histamine receptors

Histamine and H-1 receptor agonists, but not H-2 receptor agonists can stimulate increases in rat mast cell cyclic AMP suggesting that the mast cell has an H-1 receptor linked to adenylate cyclase. The histamine receptor on rat mast cells was characterized employing ^{3}H-pyrilamine binding. Mast cells have an H-1-type receptor as suggested by three lines of evidence: 1) pyrilamine, but not cimetidine, binds to mast cells, 2) pyrilamine, diphenhydramine, and tripelelanamine (all H-1 antagonists) displace pyrilamine binding while cimetidine is eighty-five times less active, and 3) H-1 agonists are five times as effective at displacing pyrilamine as are H-2 agonists. The receptor is a low affinity (K_D = 250 nM), high capacity (3.9 x 10^6 receptors/cell) type which is unusual in that most H-1 receptors are linked to guanylate not adenylate cyclase.

d. Opiate receptors

In a search for non-immunologic mast cell secretagogues, the opiates morphine and codeine, and the synthetic analgesic meperidine were examined. Rat cutaneous mast cells responded to morphine > codeine and not at all to meperidine with histamine release, but very large concentrations were required. Rat peritoneal mast cells also released histamine in response to the opiates both *in vivo* and *in vitro* but excessive amounts of agents were required. Indeed, the doses of both morphine and codeine required to induce histamine release were found to cause trypan blue dye uptake, indicating cytoxicity.

Human mast cells are 60-1000 times more sensitive to the opiates than rats, respond to all three agents equipotently ($\geq$ 5-10 μg injected), have specific opiate receptors as reflected in the capacity of naloxone to prevent morphine-induced skin test positively, and release histamine as shown by the ability of hydroxyzine to blunt the responses. Therefore, human mast cells differ from rat mast cells in the presence of an opiate receptor.

3. Clinical studies

a. Histamine responses.

Histamine infused intravenously causes flushing, headaches, tachycardia, and hypotension. Pretreatment of subjects with H-1 and/or H-2 antihistamines was used to determine which receptor(s) was involved in each response: both H-1 and H-2 receptor antagonists were required to prevent the flush, headache, and hypotension. H-1 receptor antagonists reduced the tachycardia, but H-1 and H-2 antagonists in combination were even more effective.

Plasma histamine levels were measured during the infusions of histamine; a dose-related increase was appreciated. Plasma histamines of 1.6 ng/ml were produced by infusing 0.1 μg histamine/kg/minute. Symptoms were elicited at 1.5-2 ng histamine/ml. Tachycardia appeared to be the most sensitive clinical parameter of histamine infusion. Pretreatment of subjects with both receptor antagonists revealed that the threshold for histamine-induced headaches, flushing, hypotension, and tachycardia were increased three- to four-fold. The use of H-1 and H-2 receptor antagonists to prevent and treat systemic histamine release may have major clinical implications.

b. Assay of histamine

The assay of histamine in urine currently involves comparison of diamine oxidase (DAO) digested and non-DAO-exposed samples followed by ion-exchange chromatography, extraction, and OPT condensation. Whereas the procedure is precise and accurate, the n-methyl transferase (NMT) radioenzyme assay has the advantage of speed and reduced expenses. Therefore, we developed the NMT assay for urinary histamine. Urine contains inhibitors of NMT which interfere with this enzyme activity. However, simple extraction into chloroform selectively removes histamine from these inhibitors and the extractant can be assayed for histamine. Confirmation of the chloroform extractant's identity as histamine was confirmed by DAO-digestion. The normal level of urine histamine by NMT assay is currently being determined.

Plasma histamine is < 1 ng/ml. However, the NMT assay is sensitive only to 1-2 ng/ml. Therefore, the NMT assay was modified to increase its sensitivity. The current assay employed involves single isotope labeling (^{3}H-S-adenosyl methionine), a micro procedure (final volume of 40 μl), and final separation by thin layer chromatography. Our preliminary results indicate that this assay will be sensitive to 100-250 pg/ml (ten times more sensitive than the old assay procedure).

Significance to Biochemical Research and the Program of the Institute

Increased understanding of the mechanisms of immediate hypersensitivity and its control may eventually allow improved therapy of the 44 million Americans with allergic rhinitis, the nine million with asthma, and the 22 million with urticaria. The model system employed permits sophisticated analysis of many of the aspects involved *in vivo*. Unquestionably, our analysis of mucous secretion will enable us to approach clinical problems of bronchorrhea (asthma, cystic fibrosis, chronic bronchitis) with a greater understanding, as well as permitting *in vitro* analysis of new modalities of therapy.

The observation that mast cell granules may elicit delayed inflammatory responses may provide the mechanism for explaining certain long-observed, but hitherto poorly-understood, clinical problems such as: the epithelial destruction accompanying asthma, the chronic inflammation associated with perennial rhinitis, the hyperreactive airways disease occurring in asthma and delayed responses seen with allergy skin tests. Isolation of a low molecular weight molecule eliciting this late-phase response implies potent chemotactic properties, and should allow studies of the cellular mechanisms of chemotaxis employing a biologically-relevant molecule.

Our search for mast cell secretagogues has just begun and the possibility that endogenous opiates (enkephalins) may act as such is exciting. The exact role that opiates play in allergic phenomena is unclear as yet, but has major clinical potential.

The confirmation that mast cell granule membrane receptors may be transferred to the cell membrane suggests a mechanism of replenishment of cell membrane receptors during exocytosis. The finding that the mast cell has an H-1 but not H-2 receptor reduces the likelihood that histamine "feeds back" to turn off mast cell degranulation as has been suggested for basophils.

Improved assays for plasma and urine histamine will be useful in assessing the role mast cells play in pathologic and physiologic abnormalities. Having these assays available has led to clinical studies of patients with anaphylaxis which are certain to result in increased clinical understanding and treatment. Along those lines, the use of H-1 and H-2 receptor antagonists will be useful in the prophylaxis of anaphylaxis as well as the treatment of anaphylaxis itself.

In summary, the Allergic Diseases Section is approaching immediate hypersensitivity at several levels with interests in both clinical and basic areas. This approach is providing a mileau in which results are rapidly transmitted from the laboratory bench to the clinic, and from which a finer appreciation of disease processes is evolving. The direction in which the lab is headed should continue to translate basic observations to enhanced clinical practice.

PUBLICATIONS

1. Kaliner M, 1980. Mast cell-derived mediators and bronchial asthma. In: Airway Reactivity, F. E. Hargreave, ed. Astra Scientific Publications, Ontario, Canada, p 175-188.

2. Smith L J, Shelhamer J H and Kaliner M, 1980. The cholinergic nervous system and immediate hypersensitivity. II. An analysis of pupillary responses. J. Allergy Clin. Immunol. 66:374-378.

3. Myers G, Donlon M and Kaliner M, 1981. Measurement of urinary histamine: development of methodology and normal values. J. Allergy Clin. Immunol. 67:305-311.

4. Summers R, Sigler R, Shelhamer J H and Kaliner M, 1981. Effect of infused histamine on asthmatic and normal subjects: comparison of skin test responses. J. Allergy Clin Immunol. 67:456-464.

5. Marom Z, Shelhamer J H and Kaliner M, 1981. The effects of arachidonic acid derivatives on the release of mucous glycoproteins from human airways. J. Clin. Invest. 67:1695-1703.

6. Shelhamer J H, Marom Z, and Kaliner M, 1980. Immunologic and neuropharmacologic stimulation of mucous glycoprotein release from human airways in vitro. J. Clin. Invest. 66:1400-1408.

7. Oertel H and Kaliner M, in press. The biologic activity of mast cell granules. III. Purification of inflammatory factors of anaphylaxis (IF-A) responsible for causing late-phase reactions. J. Immunol.

8. Kaliner M, in press. Asthma—pathophysiology and pharmacodynamics. Primer on Immunologic and Allergic Diseases.

9. Metcalfe D D and Kaliner, in press. The role of prostaglandins in allergic inflammation. In: Cellular Functions in Immunity and Inflammation, ed. by J. Oppenheim, D. Rosenstreich, and M. Potter. Elsevier North Holland, New York.

10. Kaliner M, Shelhamer J H, Davis P B, Smith L J and Venter J C. Autonomic nervous system abnormalities allergy. Ann. Int. Med.

11. Kaliner M, in press. Allergies: Medicine for the Layman. GPO.

12. Metcalfe D D and Kaliner M, in press. The mast cell and basophil. In: Cellular Functions in Immunity and Inflammation. ed. by J. Oppenheim, D. Rosenstreich and M. Potter. Elsevier North Holland, New York, New York.

13. Kaliner M, 1981. Mast cell-derived mediators. Proc. NE Allergy Soc. 2:28-33.

14. Wescott S and Kaliner M, in press. The effects of histamine and PGD_2 on rat mast cell cyclic AMP and mediator release. J. Allergy Clin. Immunol.

15. Shelhamer J H and Kaliner M, in press. Recent advances in pharmacologic management of allergic diseases. In: Current Perspectives in Allergy, E. J. Goetzl and A. B. Kay, eds. Churchill Livingston, Edinburgh.

16. Fraser C, Venter J and Kaliner M, submitted. Autonomic abnormalities and autoantibodies to beta-adrenergic receptors.

17. Kaliner M, Shelhamer J H and Ottesen E, submitted. Effects of infused histamine: correlation of plasma histamine levels and symptoms.

18. Bach M K, Brashler J R, Morton D R, Steel L K, Kaliner M A and Hugli T E, in press. Formation of leukotrienes C and D and pharmacologic modulation of their synthesis. Advances in Prostaglandin and Thromboxane Research.

19. Marom Z, Shelhamer J H, Bach M K and Kaliner M, submitted. Slow-reacting substances (LTC_4 and LTD_4) increase the release of mucus from human airways in vitro.

20. Shelhamer J H, Marom Z, Sun F, Bach M K and Kaliner M, in press. The effect of arachinoids and leukotrienes on mucous secretion in vitro. Chest.

21. Lemanske R F and Kaliner M, in press. The biology of mast cell secretion and its pharmacologic modulation. In: The Reticuloendothelial System: A Comprehensive Treatise, Vol. 5. Plenum Publishing Co., NY.

22. Steel L and Kaliner M, in press. Prostaglandin-generating factor of anaphylaxis: identification and isolation. J. Biol. Chem.

23. Wescott S and Kaliner M, submitted. Mast cell histamine H-1 receptors.

24. Bowman S and Kaliner M, submitted. Do mast cells have opiate receptors?

25. Lemanske R and Kaliner M, submitted. The biologic activity of mast cell granules. IV. The effects of eosinophilia on late-phase reactions.

26. Steel L S and Kaliner M, submitted. Prostaglandin-generating factor of anaphylaxis: characterization of activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00188-02 LCI

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Rapid Diagnosis of Infections by Enzyme-linked Immunoabsorbent Assays

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Stephen E. Straus	Senior Investigator, Medical Virology Section, LCI, NIAID

COOPERATING UNITS (if any) J. E. Bennett (LCI, NIAID), R. Brown (LCI, NIAID), P. Pizzo (LPO, NCI), B. Murphy (LID, NIAID), S. Rennard (LDBA, NIDR), P. Charache, and R. Yolken (Johns Hopkins University).

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Medical Virology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0		

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project Terminated.

ELISAs have been successfully developed for influenza and adenovirus antigens and for detecting type specific pneumococcal antibody. The adenovirus and pneumococcal tests continue to be routinely used in the LCI.

Publications:

1. Berg, R. A., S. I. Rennard, B. R. Murphy, R. H. Yolken, R. Dolin, S. E. Straus: New enzyme immunoassays for measurement of influenza A/Victoria/3/75 virus in nasal washes. Lancet 1: 851-853, 1980.

2. Rennard, S. I., R. Berg, G. R. Martin, J. M. Fiodart, P. G. Robey: Enzyme-linked immunoassay (ELISA) for connective tissue components. Anal. Biochem. 104: 205-214, 1980.

3. Berg, M.A., A. W. Maters, P. Charache, and S. E. Straus: Use of ELISA. Inhibition technique for typing *Streptococcus pneumoniae*. J. Clin. Micro. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00189-03 LCI

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Clinical and Biochemical Studies of Human Enteral Adenovirus Infection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Stephen E. Straus	Senior Investigator, Medical Virology Section, LCI, NIAID
H. Takiff	Clinical Associate, Medical Virology Section, LCI, NIAID

COOPERATING UNITS (if any) A.Z. Kapikian (LID, NIAID), H.S. Ginsberg (Columbia University), C. Garon, J. Rose (LBV, NIAID), R. Yolken (Johns Hopkins), J. Esparanza (Caracas, Venezuela).

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Enteral adenoviruses are a recently discovered group of agents which cause gastroenteritis in infants. They differ from all other known human adenoviruses in their inability to be propagated in tissue culture. During the past year we have continued to obtain specimens from sources around the world. Stool specimens have been characterized by enzyme immunoassay, electronmicroscopy and propagation in tissue culture. These agents have been studied extensively to determine the biological basis for their inability to grow in tissue culture. We have learned that these agents induce an abortive infection which is abruptly terminated at a very early step of the virus growth cycle in vitro. Using these findings, we have developed a successful strategy to complement the block to the virus replication and succeed for the first time in permitting the efficient replication of these viruses in cell culture. Enteral adenoviruses grown in culture for the first time have now been analyzed and characterized extensively in terms of their polypeptides, the structure of the genomes, and their antigenicity.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

1) To define the clinical spectrum of illness induced by enteral adenoviruses in both naturally occurring and experimental infections.

2) To define the biochemical properties of these adenoviruses and their relation to other respiratory adenoviruses.

3) To develop methods of promoting the growth of these agents in tissue culture so that further studies will be simplified.

Methods Employed:

1) Source of viral agents: Stool specimens believed to contain adenoviruses have been obtained in collaborative efforts from natural infections in infants in Washington, D.C., Baltimore, Bangladesh, and Venezuela.

2) Identification of agents: Presumptive agents are characterized by enzyme immunoassays performed here or in Baltimore utilizing antisera developed in this laboratory. Electronmicroscopy is performed by Dr. Kapikian to identify and quantitate the adenovirus content of specimens. The specimens are then innoculated and blind passaged in primary and continuous human cell lines which are then examined for adenoviral cytopathic effects over four to six week period. Agents which by electronmicroscopy have the structure of adenoviruses, react as such immunologically, and fail to propagate in conventional cell lines are considered candidate enteral agents.

3) In vitro tissue culture studies: Human cell lines are innoculated with enteral adenovirus containing material and examined for cytopathic effect and the production of late viral proteins by immunofluorescence microscopy.

4) Biochemical studies: Enteral adenoviruses which have now been grown in the adenovirus type 5 transformed human embryonic kidney cell line (293 cells) have been extensively studied. We have further studied block replication of these passaged isolates in conventional cell lines. Virus DNA synthesis has been studied by labeling, Hirt extractions and sucrose gradient analyses. We have characterized restriction endonuclease cleavage patterns of a number of isolates and have performed blot hybridization studies to indicate the extent and areas of homology to adenovirus type 2.

5) Clinical Studies: Using our efficient cell culture technique, we have been cultivating isolates from natural outbreaks of enteral adenovirus-induced diarrheal illnesses and have begun to perform molecular and sero-epidemiologic studies on the various strains.

Major Findings:

To date we have established sources for enteral adenovirus specimens, developed the means of unequivocally identifying the agents, have arrived at an understanding of the inability of these viruses to be cultured in conventional cell lines, and have most recently succeeded in developing a cell culture system to efficiently grow these viruses. Our biochemical studies have successfully defined a number of the structural characteristics of the virus and its genome.

Proposed Course:

During the next year we propose to complete our restriction endonuclease analysis of a variety of isolates of enteral adenoviruses and to physically map the viral genome. We hope to then direct our attention to the molecular basis for the restriction of these viruses in conventional cell lines and attempt to arrive at some understanding as to how these viruses grow in the human host.

Publications:

1. Takiff, H.E., Straus, S.E., Kapikian, A.Z., Rose, J.A., Lewis, A.M.,Jr., Ginsberg, H.S., Levine, A.H.: Studies of the replication of enteral adenoviruses. Presented at the American Society for Microbiology Meeting, Dallas, Texas; March, 1981.

2. Takiff, H., Straus, S., and Garon, C.: Characterization of enteral adenoviruses propagated in 293 cells. Abstract presented at the ICRF Meeting on Tumor Viruses, Cambridge, England; July, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00192-03 LCI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Studies on Immediate Hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. D. Metcalfe	Senior Clinical Investigator	LCI NIAID
Other:	A. Saavedra-Delgado	Medical Officer	LCI NIAID
	P. V. Subba-Rao	Visiting Scientist	LCI NIAID

COOPERATING UNITS (if any)

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Laboratory of Clinical Investigation

SECTION
Allergic Diseases Section

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NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3 6/12	PROFESSIONAL: 2 6/12	OTHER: 1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A proteoglycan heparin has been identified and characterized in mouse mast cells grown in vitro in lymphocyte-dependent culture systems. A histamine-containing cell cloned from mouse bone marrow has been identified as an atypical mast cell which contains a unique proteoglycan chondroitin 4-sulfate. The function of heparin may in part be to form a protease-resistant heparin granule which persists in tissue after release from mast cells until phagocytosed, especially by connective tissue fibroblasts. Following phagocytosis of these heparin granules, fibroblasts respond with increased enzyme secretion.

Typical and atypical gastrointestinal mast cells have been enumerated throughout the rat gastrointestinal system and correlated with tissue histamine. A protocol to study food allergy has been developed.

Patients participating in the mastocytosis protocol reveal a high incidence of Type IV hyperlipoproteinemia, bone involvement, and cardiac disease.

Project Description

Objectives

We have three primary objectives. First, to identify and characterize mast cell and basophil proteoglycans, to determine what factors influence their synthesis and degradation, and to determine their pathophysiologic function. Second, to develop *in vivo* methods to study the pathophysiologic role of human mast cells in disease. Third, to study immediate hypersensitivity reactions in the gastrointestinal tract with emphasis on food allergy.

Methods Employed

1. Studies on mast cell and basophil proteoglycans

Mast cells and basophils grown in culture are radiolabeled using radioactive materials selectively incorporated into specific structural features of the proteoglycan molecules. These proteoglycans are extracted and purified using both ion-exchange chromatography and gel filtration. The structure of the molecule can be further elucidated by a variety of biochemical techniques. The quality and quantity of proteoglycan molecules produced gives information on synthesis. Molecules isolated as a part of the mast cell granule, or solubilized in high salt, can be used both for *in vitro* examination of their biologic function and their catabolism.

2. Studies on the pathophysiologic role of the mast cell

Two current clinical protocols are designed to study human mast cells. In the first protocol, patients with mastocytosis who have multiple organ systems infiltrated with mast cells are examined as to their response to mediators which affect mast cell degranulation or which block the action of mast cell-derived mediators (histamine). By close clinical evaluations and follow-up, normal physiologic roles of the mast cell can be inferred from the consequences of excess mast cell number. In the second protocol, individuals with cold urticaria have an extremity challenged with ice water. This results in the release of mast cell substances which can be collected by venipuncture, and subsequently identified and characterized as to possible pathophysiologic roles.

3. Studies on food hypersensitivity and the role of the gastrointestinal mast cell

A clinical protocol on food allergy has been approved where history and diagnostic tests for food allergy may be correlated with blinded food challenge. Mediators released during challenge are being identified. This mediator identification will allow a rational selection of drugs for treatment of food allergy. An animal model to study gastrointestinal mast cells has been developed and the response of mast cells both to oral medications and oral protein challenge after systemic immunization is being determined.

Major Findings

1. Studies on mast cell and basophil proteoglycans

Mouse mast cells grown *in vitro* under the influence of lymphocytes have been shown to synthesize proteoglycan heparin and chondroitin sulfates. This observation suggests that proteoglycan heparin, heretofore shown only in rat mast cells, may be the native molecule in many species. In this instance, the differentiation of the mouse mast cell and the simultaneous synthesis of heparin were dependent upon lymphocyte-derived stimuli. Using cloning techniques, a second histamine-containing cell has been grown from bone marrow in lymphocyte-dependent culture systems. Such a cell has been called both a mast cell and a basophil. However, we have shown that this cell lacks heparin, containing instead a unique proteoglycan chondroitin 4-sulfate. This finding, as well as morphologic data, strongly suggests that this bone marrow-derived cell is an atypical mast cell found particularly in the gastrointestinal wall lamina properia. Thus, proteoglycan identification has been added as a criteria of mast cell identification, a new cell-associated proteoglycan reported, and a cell line identified which may be of interest in future studies in gastrointestinal mast cell function.

Proteoglycan heparin is protease resistant, highly charged, and forms the scaffolding of the mast cell granule. Its characteristics allow certain proteins to remain complexed to it after granule discharge in a loose configuration. This extracelluar residual granule persists in tissues until apparently phagocytosed by fibroblasts and infiltrating phagocytic cells. We have been exploring the possibility that discharged mast cell granules, whose structure depends upon heparin, can influence fibroblast function. We have developed *in vitro* primary rat fibroblast monolayers and are studying the effect of mast cell granules upon these preparations. We have shown that fibroblasts do, indeed, phagocytose mast cell granules, and may increase the secretion of enzymes as a response to this stimulus. One might consider this phenomenon a means by which mast cells can influence connective tissue in normal and disease states, influencing both tissue repair and degradation.

2. Studies on the pathophysiologic role of the mast cell

Initial screening of individuals who are candidates for the mastocytosis study has shown an expected high incidence of bone disease as manifested by abnormal bone x-rays and scans, and clinically by bone pain. An unexpected finding was an unusually high incidence of Type IV hyperlipoproteinemia (four out of five patients), and two documented cases of mitral value prolapse. The former observation is counter to a widely held impression that mast cells protect against athrosclerosis by virtue of their heparin, which when given IV has a lipid-clearing effect. However, this observation does agree with our *in vitro* observations that heparin granules are rapidly phagocytosed by fibroblasts, in which case heparin would rarely reach the vascular compartment. Further, no evidence of heparin has been found in the blood stream of individuals with cold urticaria of the cold challenge of an extremity performed on the cold urticaria protocol. The etiology of the Type IV hyperlipoproteinemia is unknown, but may have to do with the influence of mast cells upon the liver or upon peripheral tissues. The etiology of the mitral value prolapse is unknown.

3. Studies on food hypersensitivity and the role of the gastrointestinal mast cell

Using the rat to study the role of the gastrointestinal mast cell, we have enumerated both typical and atypical mast cells throughout the gastrointestinal system, showing a majority of the atypical mast cells to be located in the small intestine. Further, we have correlated tissue histamine content in all regions to atypical and typical mast cell number. This knowledge of the relative frequencies of both mast cell types to the mediator histamine forms the basis for further experiments to determine those factors that regulate their numbers and stimuli that lead to their degranulation.

A clinical protocol to study food hypersensitivity has been approved which will correlate history of food intolerance with diagnostic tests, oral food challenge, and mediator release. It is hoped that this protocol will lead to a basic understanding of the pathophysiology of a food-induced allergic reaction. This basic protocol would then be extended to explore avenues of treatment.

Significance to Biomedical Research

The isolation, identification, and characterization of proteoglycans in those cells involved in immediate hypersensitivity contributes to the understanding of the pathophysiologic role of the mast cell. By virtue of their unusual characteristics, including large size and high negative charge, proteoglycans appear to have the capacity to not only function in the intracellular storage of inflammatory mediators, but to influence events that follow mast cell degranulation. The protocols to examine human mast cell function in cold urticaria and mastocytosis allow observations made on mast cell function *in vitro* to be applied to human mast cell-mediated disease. Specifically in mastocytosis, the hope is to determine the best method of treatment in this mast cell disorder. The extension of mast cell studies into gastrointestinal hypersensitivity will lead directly to a detailed examination of the complex area of food allergy.

Proposed Course

We intend to characterize human mast cell proteoglycans as our *in vitro* techniques for growing these cells improve, and to study factors controlling their synthesis and degradation. We will concentrate on the influence of heparin granules upon surrounding cells. Mastocytosis and cold urticaria protocols will continue to yield information on the *in vivo* function of human mast cells. The food allergy protocol will develop into a major endeavor as selected patients are studied and treated for their complaints.

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2. Metcalfe D D, Soter N A, Wasserman S I and Austen K F: Identification of sulfated mucopolysaccharides including heparin in the lesional skin of a patient with mastocytosis. J. Invest. Derm. 74:210-215, 1980.

3. Shelhamer J H, Metcalfe D D, Smith L J and Kaliner M: Abnormal beta adrenergic responsiveness in allergic subjects: Analysis of isoproterenol-induced cardiovascular and plasma cyclic AMP responses. J. Allergy and Clinical Immunol. 66:52-60, 1980.

4. Metcalfe D D, Smith J A, Austen K F and Silbert J E: Polydispersity of rat mast cell heparin: Implications for proteoglycan assembly. J. Biol. Chem. 255:11753-11758, 1980.

5. Kazatchkine M D, Fearon D T, Metcalfe D D, Rosenberg R D and Austen K F: Structural determinants of the capacity of heparin to inhibit the formation of the human amplification C3 convertase. J. Clin. Invest. 67:223-228, 1981.

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7. Metcalfe D D and Kaliner. The role of prostaglandin in allergic inflammation. In Cell Function Immunity and Inflammation, ed. by J. Oppenheim, D. Rosenstreich, and M. Potter. Elsevier North Holland, New York, in press.

8. Metcalfe D D and Kaliner M: The Mast Cell and Basophil. In Cell Functions in Immunity and Inflammation. Ed. by J. Oppenheim, D. Rosenstreich and M. Potter. Elsevier North Holland, New York, New York, in press.

9. Metcalfe D: Food Allergy. In Primer on Immunologic and Allergic Diseases, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00155-06 LCI

PERIOD COVERED October 1, 1979 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Phagocytic Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

Phagocyte activation has been studied using membrane potential sensitve fluorescent probe, surface charge and ultrastructural techniques in leukocytes from normal subjects and patients with abnormal phagocytes and using pharmacologic probes such as histamine and Vitamin K. The data indicate that limited secretion of specific granules, which accompanies chemotaxis, is associated with increased cell adhesiveness and increased availability of chemoattractant receptors. Limited secretion may be required for neutrophil margination in vivo. A patient with specific granule deficiency has been indentified and his neutrophils support these concepts.

Studies of the two populations of neutrophils we had identified previously have been extended. Neutrophil heterogeneity of elicited changes in membrane potential to chemotactic factors have been demonstrated using the fluorescent activated cell sorter and the probe di-O-C5(3). Neutrophil function studies indicate that some "acquired" defects of neutrophil function represent redistribution of neutrophil populations rather than intrinsic cell defects. In other studies of the pathogenesis of abnormal chemotaxis during pregnancy an acquired neutrophil defect with abnormal neutrophil chemoattractant receptors has been identified.

Project Description

Objectives:

1) Study the mechanism of leukocyte chemotaxis.

2) Study the phenomenon of deactivation of leukocyte chemotaxis and to relate this to the pathophysiology of leukocyte dysfunction syndromes.

3) Develop new micro techniques to quantitate chemotaxis, spreading of living phagocytes, and assessment of phagocyte membrane receptors.

4) Study the role of cations in leukocyte activation with particular emphasis on the utilization of indirect probes of membrane potential to study the relationship between changes in membrane potential and initiation of neutrophil motility, phagocytosis, degranulation and superoxide generation.

5) Study neutrophil subpopulations in normal subjects, in patients with neutrophil dysfunction, and to develop an animal model to study the kinetics of clearance of neutrophil populations *in vivo*.

6) Study the mechanism of neutrophil margination and aggregation at capillary beds prior to cell migration into tissues.

7) Study the mechanism of mobilization and secretion of neutrophil lysosomal granules, study the relationship of the secretory process to neutrophil function and dysfunction and to study how neutrophil secretory products may influence homeostasis.

8) Characterize and define the mechanism of abnormal neutrophil chemotaxis during pregnancy.

9) Study the effects of pharmacologic agents on leukocyte function *in vitro* and *in vivo*.

10) Assess the effect of histamine on neutrophil function.

11) Monitor white blood cell transfusions in humans and to determine the indicated frequency of delivery of transfusions and the efficacy of transfused cells.

12) To study macrophage granulomas *in vitro* and to understand why patients with chronic granulomatous disease of childhood develop granulomas.

Methods Employed

Peripheral blood leukocytes were separated from heparinized whole blood by dextran sedimentation and Hypaque-Ficoll separation. Chemotaxis was evaluated using either a radioassay employing ^{51}Cr labeled leukocytes and a double micropore filter system or a single filter measuring the distance migrated into the filter by the population of responding cells. Quantitation of cell locomotion in the later system utilized an image analyzer and computor assisted plotter. Cell adherence was measured by quantitating the number of leukocytes adhering to plastic surfaces or to nylon wool columns. Phagocytosis, bactericidal capacity, nitroblue tetrazolium dye reduction, hexose monophosphate shunt activity, superoxide generation and hydrogen peroxide formation were measured using standard techniques. PMN receptors for the chemoattractant f-met-leu-phe were quantitated as published previously. New methods were developed to monitor the availability of Fc and C3b receptors using EA rosetting techniques.

The fluorescent probe dipentyloxacarbocyanine di-O-C5(3) and the radioisotope trimethylphosphonium ions were used as indirect probes of membrane potential in neutrophil suspensions. The fluorescent activated cell sorter was used to monitor individual cells. Leukocyte surface charge was measured with a Zeiss cytopherometer. Intracellular calcium was localized in human neutrophils by the intracellular precipitation of pyroantimonate anion followed by microprobe analysis and studies with the metal chelators (EDTA and EGTA).

The cytoskeleton of polymorphonuclear leukocytes was studied during conditions of chemotaxis and chemokinesis using 0.45 μm micropore filters. These small filters impede leukocyte migration but permit pseudopod penetration and a fixed orientation of leukocytes is thereby established. Orientation of living cells was monitored by well established techniques. Tyrosylation of the α-chain of tubulin was studied as described previously. Secretion of leukocyte granule enzymes was monitored by standard spectrophotometric assays. Polymorphonuclear leukocyte granules were separated and fractionated using sucrose gradient techniques and various enzyme markers were utilized to identify granule types.

Neutrophils were fractionated into subpopulations based on their ability to rosette IgG coated erythrocytes. In some studies, in normal human volunteers, the effects of intravenous endotoxin on neutrophil subpopulations was examined. Neutrophil heterogeneity was also identified in the fluorescent activated cell sorter using the probe di-O-C5(3) and PMN activated with the chemoattractant f-met-leu-phe.

Major Findings

1. Technologic advances have been made for quantitating leukocyte chemotaxis, bactericidal activity and availability of Fc and C3b receptors. Using an Optomax image analyzer the amount of time required to quantitate the number of cells migrating a distance into a micropore filter or the number of bacteria on a pour plate has been reduced by 90%. The image analyzer, in conjunction with a new microchamber of chemotaxis, enables 48 determinations in a single filter using volumes of cells and stimuli 1/20 used previously. This has facilitated greatly the number of samples processed each day and reduced greatly the amount of blood that has to be obtained from subjects. In addition, the image analyzer has made possible a quantitative assay of leukocyte spreading. Preliminary studies indicate the image analyzer will make possible the tracking of moving cells thereby providing quantitative information on initial rates of locomotion (Alling, Gallin).

A micro-method for determining percentages of rosette forming leukocytes has also been developed. The method requires approximately 50μl of blood to determine rosetting percentages (of either Fc or C3b rosetting cells) and allows frequent, repetitive rosette percentage determinations during intravenous survival studies.

It could also be applied to clinical screening of newborn infants (Buescher, (Gallin).

2. Recent work relating the preferential release of specific granules from neutrophils by secretagogue stimulation to enhanced binding of the chemoattractant f-met-leu-phe has demonstrated a positive correlation between the degree of specific granule exocytosis (as measured by lysozyme release) and the degree of surface bound f-met-leu-phe for secretion levels up to 20% of the total cellular lysozyme. Expression of this enhanced chemoattractant binding does not require new protein synthesis, is not affected by the specific granule constitutents lysozyme or lactoferrin, nor is it due to a secondary modification of the plasma membrane by the toxic oxygen metabolites normally generated during secretagogue stimulation of the cells. Neutrophils demonstrated to enhance their number of surface receptors after secretagogue exposure are also more responsive in f-met-leu-phe elicted membrane potential changes and superoxide generation. Studies in a patient whose neutrophils are deficient in specific granules and specific granule markers support these concepts. The studies in this patient include the demonstration of decreased binding of the chemoattractant f-met-leu-[^{3}H]phe relative to a group of normal control cells as well as a quantitative defect in receptor mobilization after stimulation with a secretagogue (Fletcher, Gallin).

Studies of cell activation using normal volunteers are presently underway to evaluate the role of granule exocytosis in the cellular changes which accompany neutrophil aggregation and margination in vivo in order to correlate such processes with previously demonstrated mechanisms in vitro. In particular, evidence for early modulation of the availability of chemoattractant receptors at the cell surface is being sought. Related studies have been begun to investigate what effects the standard neutrophil isolation procedure may have on surface expression of chemoattractant receptors in an attempt to more accurately determine the magnitude of chemoattractant receptors which neutrophils possess on their surfaces while circulating in the blood stream before they have been activated (Fletcher, Gallin).

3. The number of receptors for the chemotactic peptide f-met-leu-phe on neutrophils which have been chemotactically deactivated was examined. The data indicate that chemotactic deactivation towards more than one chemotactic stimulus is associated with an increase in f-met-leu-phe receptors, while preferential chemotactic deactivation towards f-met-leu-phe alone is associated with a decrease in f-met-leu-phe receptors. This work provides insight into the mechanism of chemotactic deactivation, a potentially important mechanism of neutrophil motility regulation (Donabedian, Gallin).

4. Work is underway to examine the status of f-met-leu-phe receptors on neutrophils which are actually in the process of chemotaxis. Attempts will be made to guage the importance of regulation of receptor number in the response to a chemoattractant and to judge the effect of chemotactic deactivation on these processes (Donabedian, Gallin).

5. Vitamin K derivatives (Vitamin K_3 and Vitamin K_5) are effective inhibitors of neutrophil spreading, locomotion, phagocytosis and killing without affecting receptor availability, hexosemonophosphate shunt activity or lactate production. Vitamin K appears to exert its effect by Vitamin K epoxide oxidation of vital sulfhydryl groups and possibly Vitamin K carboxylation of γ carboxy glutamic acid,

a calcium sequestration system. The Vitamin K effects on the neutrophil also include inhibition of elicited membrane potential changes, stimulation of calcium release, accumulation of submembraneous calcium and inhibition of the assembly (morphologic analysis) and the tyrosylation of the α-chain of tubulin. The possibility Vitamin K derivatives possess select antiinflammatory activities is being explored (Gallin, Seligmann, Cramer, Nath, Schiffmann).

6. The use of indirect probes to study cell membrane potential has been continued and expanded. Other probes have been evaluated and as a result two fluorescent probes have become the primary choice for routine use. One has fluorescein fluorescence, the other rhodamine fluorescence. Neither effects neutrophils adversely (whereas other probes studied did have adverse effects) (Seligmann, Gallin).

7. The effects of antibiotics on the uptake of di-0-C5(3) by bacteria has been studied. The most striking observation is that the cell wall of certain bacteria (gram negative) prevents di-0-C5(3) equilibration with the bacteria. Treatment with antibiotics which attack the cell wall enable the dye to equilibrate rapidly. This may be useful in the future in the rapid detection of antibiotic sensitivity (Seligmann, MacLowry, Gallin).

8. The determination of the effects of a wide variety of venoms on neutrophil function have been initiated using neutrophil membrane potential as a screening test of venom action. We are in the process of purifying a component of the European wasp venom which we have demonstrated causes neutrophils to degranulate and which depolarizes PMN membrane potential (Seligmann, Pisano, Gallin).

9. Studies of di-0-C5(3) uptake in neutrophils using the fluorescent activated cell sorter indicate that a population of neutrophils exhibit a uniform resting membrane potential and respond uniformly to several stimulii such as phorbol myristate acetate and A23187. However, the response of neutrophils to the chemoattractant f-met-leu-phe is heterogeneous with only about 60% of the cells exhibiting a large potential change. The physiologic significance of this neutrophil heterogeneity is being explored and may relate to neutrophil heterogeneity described below (Seligmann, Chused, and Gallin).

10. Assessment of chemoattractant elicited membrane potential changes using indirect probes indicate that neutrophils from patients with chronic granulomatous disease (CGD) have a major abnormality (decreased response). Neutrophils from patients with other phagocyte defects do not show any abnormality of elicited membrane potential changes. Studies in the fluorescent activated cell sorter indicate the defect in CGD PMNs is of all the patients' cells. Studies of calcium, potassium, sodium and chloride fluxes are currently underway to further characterize the basis of the defect in CGD neutrophils (Seligmann, Gallin).

11. Pregnancy is associated with a dramatic increase in the number of circulating neutrophils. We have shown that neutrophil chemotaxis to standard stimuli including f-met-leu-phe is significantly depressed during pregnancy reaching a nadir at 26-30 weeks of gestation and partially reversing before term. Moreover, superoxide production to f-met-leu-phe is markedly reduced. Preliminary studies suggest a decrease in the specific binding of f-met-leu-[^{3}H]phe to neutrophils from pregnant woman is associated with the abnormal chemotaxis of their cells. The possible role of hormonal changes in the production of these functional alterations is currently

being explored. Such alterations may contribute to the increased morbidity from certain infections seen during pregnancy, to the establishment of intrauterine infection and to the known amelioration of certain inflammatory diseases, such as rheumatoid arthritis, during pregnancy (Cotton, Gallin).

12. Abnormal PMN function in cells from patients with the Chediak-Higashi syndrome is associated with a 300 fold increase in the tyrosylation of the α-chain of tubulin. This represents the first biochemical evidence for abnormal microtubule metabolism in a patient with phagocyte dysfunction (Nath, Gallin).

13. We have previously demonstrated that neutrophils can be divided into two subpopulations based on their ability to form rosettes with erythrocytes sensitized with IgG. Those that form rosettes (Fc+) are superior in functional assays to those that do not form rosettes (Fc-). After administration of intravenous endotoxin to normal volunteers there is a decrease in the number of circulating neutrophils which are Fc+. We are currently investigating whether this decrease is due to a shift of Fc+ cells to the marginated pool and whether it is accompanied by measurable changes in the functional capabilities of the cells remaining in the circulation (enriched for Fc- cells) (Cotton, Gallin).

14. A rabbit animal model for investigating intravascular survival of neutrophil subpopulations was developed using micro-methods. Intravascular survival of ^{51}Cr tagged rabbit heterophils was followed and initial studies suggest different half-lives for (Fc+) and (Fc-) subpopulations. Further studies are underway (Buescher, Gallin).

15. Peripheral blood neutrophils exist in two pools of roughly equal size: a circulating pool and a pool of cells which is adherent to the capillary endothelium. It has long been assumed that the cells of each pool are morphologically and functionally identical. We are currently testing this hypothesis by shifting the marginated cells into the circulation in human volunteers by administration of intravenous epinephine and assaying the neutrophils so obtained for chemotaxis, rosetting of IgG coated erythrocytes, killing of bacteria, lysosomal enzyme content and superoxide production (Cotton, Gallin).

16. Work is ongoing on the clinical course and physiology of the Hyperimmunoglobulin E and Recurrent Infection Syndrome (HIE). A prospective double-blinded test of the clinical efficacy of levamisole is underway (Donabedian, Gallin).

17. A major advance in the understanding of the basis of HIE disease has been reached with the finding that mononuclear cells from HIE patients produce a factor which inhibits normal neutrophil chemotaxis. The production of this factor in vitro correlates with the presence of an in vitro chemotactic defect. This finding opens the possibility of examining the relatively untouched field of mononuclear cell regulation of neutrophil function (Donabedian, Gallin).

18. The effect of histamine on neutrophil function using histamine was well as H_1 and H_2 agonists and antagonists has been studied. It was found that both H_1 and H_2 agonists inhibit f-met-leu-phe stimulated chemotaxis, killing, secretion, superoxide production, chemiluminescence, peroxide production and membrane potential changes, but enhance the chemokinesis of neutrophils. These effects are reversed by the H_2 antagonist cimetidine. There is no affect on PMA or A23187 stimulated

functions, indicating the effect is specific for f-met-leu-phe. There was no effect of histamine on f-met-leu-[^{3}H]phe binding. The results suggest a direct action of histamine or an early common step in the specific activation mechanisms stimulated by f-met-leu-phe. The clinical implications are obvious since histamine is a primary substance released during hypersensitivity reactions and at other times during inflammation and in patients with the Hyper IgE Recurrent Infection syndrome (Seligmann, Fletcher, Gallin).

19. Peripheral blood mononuclear cells from patients with Chronic Granulomatous Disease (CGD) were cultured and observed. Formation of large multinucleated giant cells was observed in some cultures after 3-5 weeks. The mechanism of human giant cells formation in vitro in this setting represents fusion of mononuclear cells rather than nuclear replication (Buescher, Gallin).

20. White blood cell transfusions were administered to a patient with chronic granulomatous disease as part of therapy for pneumonia. Using nitroblue tetrazolium (NBT) dye reduction as a marker, intravascular survival of transfused cells was followed, as well as their appearance and persistence in the sputum. NBT reduction (superoxide dismutase and catalase inhibitable) in sputum was found in the CGD sputum and NBT + macrophages were cultured from sputum during WBC transfusions. The data indicate once a day transfusion is adequate to deliver functionally active white blood cells to inflammatory foci (Buescher, Gallin).

Significance to Biomedical Research and the Program of the Institute

The accumulation of leukocytes at inflammatory, immune and allergic sites is critical for appropriate responses. Understanding the physiologic basis for events regulating these processes will provide the basis for therapeutic manipulation.

One of the first steps in leukocyte mobilization from the blood stream is increased cell adhesiveness to the endothelium. This is followed by local leukocyte aggregation and then diapedesis. Our finding that limited degranulation and exocytosis of intracellular granules markedly enhcances these events, together with our prior observation that human pyrogen initiates these processes, provides a clue as to what controls margination and then migration of leukocytes from the blood stream. The related observation that vigorous degranulation in vitro inhibits chemotaxis suggested that some acquired chemotactic defects relate to excessive degranulation in vivo. In support of this is our previous finding that following thermal injury the severity of the acquired chemotactic defect is linearly related to the amount of intracellular lysozyme released. Clinically it is of interest that the degranulation and defective chemotaxis precedes the severe and often lethal pyrogenic infections that follow thermal injury. In addition, the identification of a patient deficient in neutrophil specific granules with recurrent infections and predicted defects in neutrophil function supports these concepts. In related studies it appears that lactoferrin, contained in neutrophil specifc granules and secreted when cells are activated, facilitates neutrophil spreading and adhesivenss. Thus the neutrophil secreted product lactoferrin may be critical for margination.

Two observations from our studies of the mechanism of the phenomena of degranulation, cell adhesiveness and chemotaxis may be particularly important to the cell biology of chemotaxis and perhaps relevant to clinical studies as well. We have shown that leukocyte adherence, aggregation and secretion may be under the modulating influence of electrostatic forces. Development of techniques for controlling these forces in vivo may have potential clinical use. Our data that neutrophil specific granules are a potential source of new cytoplasmic membrane and chemoattractant receptors is intriguing in terms of understanding the basis for membrane turnover during chemotaxis. The demonstration of abnormal chemotaxis and an abnormality in f-met-leu-phe chemoattractant receptors in neutrophils obtained from pregnant women may be particularly relevant in this regard. These studies need to be extended and explored further.

The use of fluorescent carbocyanine dyes to measure membrane potential is emerging as a rapid test of cell responsiveness that appears to be as reliable and easier to use than other indirect probes of membrane potential. Adaptation of this method of the fluorescent cell sorter provides an opportunity to look at individual cells and to better characterize and detect subpopulations of neutrophils. The demonstration of a severe abnormality of chronic granulomatous disease neutrophils using this test may provide a new simple rapid diagnostic test for this disease. In addition, the implication that the observed abnormality in chronic granulomatous disease reflects abnormal ion flux, perhaps related to abnormal activation mechanisms, provides new insights into the nature of the defect in chronic granulomatous disease.

The continuation of our studies of neutrophil subpopulations has indicated that one population of cells (PMNs with readily demonstrable Fc receptors) is particularly available for margination. We are presently extending these studies of neutrophil subpopulations to explore their kinetics of circulation, and their functional role. The identification of heterogeneity of neutrophil responses to chemoattractants in terms of elicited responses seen with the membrane potential sensitive fluorescent probe di-O-C5(3) in the fluorescent activated cell sorter has facilitated these studies. In addition, the effect of various disease states and pharmacologic agents on these two neutrophil populations in man and in animals is under study.

The demonstration of a lymphocyte product that inhibits neutrophil function in patients with the hyper IgE-recurrent infection syndrome provides a new approach to the relatively untouched field of lymphocyte regulation of neutrophil function.

The demonstration that Vitamin K derivatives have pronounced effects on neutrophil function may have important implications in terms of understanding normal neutrophil function and also may provide new approaches for pharmacologic manipulation of phagocyte function in inflammatory diseases.

Proposed Course

We plan to continue some of the studies outlined above.

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LABORATORY OF IMMUNOGENETICS
1981 Annual Report
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Annual Report of the Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, NIH, PHS, HHS, October 1, 1980 - September 30, 1981

Research Progress

The Laboratory of Immunogenetics applies modern techniques in protein structural chemistry, immunology, and immunobiology to the study of fundamental questions of immunogenetics. These questions have concerned immunoglobulin molecules and the genes encoding these molecules with emphasis on regulatory processes involved in expression of Ig genes. Studies concerning membrane antigens encoded by the major histocompatiblity complex have concentrated in the past on the murine major histocompatibility complex, but will be expanded to included these gene complexes in the rabbit and the human. Research into techniques for protein microsequencing anlaysis have emphasized gas chromatographic-mass spectrometric methods and progress is being made in this area. Hybridoma antibodies are being prepared and used in quantitative assays for various cell surface markers and in procedures for isolation membrane bound antigens.

Characterization of Histocompatibility Antigens. Products encoded at the major histocompatibility complex (MHC) determine a diversity of functions associated with immune recognition and reactions. Our work is aimed at determination of primary structures for molecules encoded at the MHC in order to better understand their function and mechanisms of action. In the past, work has concentrated on murine MHC antigens and the entire 346 residue structure of the H-2K^b antigen (Coligan) and its 99 residue subunit, beta-2-microglobulin (Gates) has been determined. In addition, major portions of the primary structures for four other H-2 antigens (D^b, K^d, D^d, and L^b) have been determined (Kimball, Maloy) and their structures have been compared to that of the K^b. On the basis of our data, a model can be constructed showing the extracellular portions of the molecule, the part that binds the membrane, and the intracellular portion of the molecule. Comparative studies of the structure show that there are discrete areas of difference which may include the sites recognized by T cells and those involved in other immune functions.

Previous study (Kimball) on rabbit histocompatibility antigens utilized molecules isolated on the basis of their association with beta-2-microglobulin (B_2-m). More recently monoclonal antibodies have been prepared against fractions of a rabbit T cell line and certain of these antibodies would precipitate a 42,000 dalton molecule from radiolabeled lysates of this cell (Wilkinson). It was shown by radioactive sequence analysis that this 42,000 molecule is similar or identical to the molecule obtained with anti-B_2-m. This identity was verified by two-dimensional electrophoresis. However, in spite of this apparent identity, the molecule associated with B_2-m and that recognized by the monoclonal antibody are serologically distinct. Studies are currently underway to determine the nature of these differences (Tykocinski). Possible alternatives include the possibility that the monoclonal antibody recognizes the conformational determinant that is not present when the chain is associated with B_2-m or that a genetic variant of the chain which exists in the cell-bound portion is being recognized by the antibody.

Studies on human histocompatibility antigens have begun with development of radioimmune assays for quantitation of HLA-A, -B, -C, and -DR antigens

(Quijano). These assays are being used to determine optimum procedures for isolation of these antigens from lymphoid cell lines (Kuo, Sachs). In collaboration with Dr. W. Biddison, NCI, NIH, the HLA-3 antigen is being studied in detail to determine whether multiple structural forms of this molecule will correspond to differences observed in recognition of A-3 positive cells by cytolytic T cells (Coligan, Marti).

Mass Spectometry for Sequence Analysis of Peptides. Work continues on the GC/MS approach to sequence analysis of polypeptides (Krutzsch). Use of a fused glass capillary column has increased the sensitivity of this method so that 50 picamoles of digestion mixture is easily detected in the GC/MS. The method has been applied to peptides from various sources including viral coat proteins and encephalins with varying degrees of success.

Genetic studies on serum proteins. Studies of rabbit immunoglobulin allotypes have utilized rabbit-mouse hybridomas and a number of new cell lines have been prepared within the past year. Improved techniques for stabilization of these hybridomas have recently allowed preparation of a cell which produces both heavy and light chains (Sogn). Characterization of the products of this and other hybridomas is well underway. Several unusual structural characteristics have been determined for the secreted products (Dreher, Kuo).

In addition, the studies on rabbit mouse hybridomas have revealed that rabbit spleen cells in culture give rise to a large cell which has a long life in culture (Sogn, Jackson). Characterization of these cells by a variety of techniques indicated that they were not adherent macrophages. Studies are underway to determine if these cells can be maintained in culture.

Studies on hamster female proteins being carried out in collaboration with Dr. John Coe of Rocky Mountain Laboratory involves primary structural detemination of this unique serum protein. Preliminary results indicate that it is structurally similar to C-reactive proteins and amyloid P components.

Rabbit Latent Allotypes. Descriptions of genetically unexpected immunoglobulins (latent allotypes) in several species suggest that the Ig gene contains more information than is normally expressed. Our studies have emphasized latent allotypes of the rabbit and recently we have attempted to determine conditions for *in vivo* induction of latent allotypes (Yarmush, Kindt). This has been possible by injection of anti-allotype antibody, followed by boosting with an appropriate antigen. Original studies used anti-streptococal antibody in the rabbit and more recently we have attempted to reproduce these studies with ovalbumin.

In conjunction with our studies on the latent allotypes, we have modified an ELISA type assay to determine rabbit allotypes (Jackson). This is an extremely sensitive assay and many of the difficulties associated with the previously used radioimmune assay have circumvented by its use.

Rabbit-mouse hybridoma cells that produce rabbit Ig chains are being used as a source of mRNA for rabbit Ig chains (Gates). The mRNA is being purified by a variety of techniques to use in the construction of DNA probes for determination of the structure of the Ig genome in the rabbit (Dreher, Emorine). An initial goal in these molecular biological studies will be to determine the number of genes that encode the rabbit kappa L chain and the rabbit V_H region.

Monoclonal Antibodies Directed against Cell Surface Proteins. Although much is known about the humoral system of the rabbit, very little is known about the cells involved in the immunity of this animal. One possible approach to studying cellular immunology is to prepare antibodies directed against subpopulations of lymphoid cells. Although a number of heterologous antisera had been used in the past, monoclonal antibodies are by far preferred for this purpose. We have immunized mice with extracts from a rabbit T cell line, prepared hybridomas, and selected their products for positive reactivity for RL-5 (Wilkinson). Twenty-eight monoclonal antibodies were selected and studied by precipitation and by surface fluorescence. In this group are several with reactivity for subpopulations of rabbit lymphoid cells including T cells and other Ig negative cells (Chusid, Wilkinson).

In attempts to prepare monoclonal antibodies directed against polymorphic HLA antigens, an antibody was found that reacts with a number of lymphoid cell lines but with no normal cells, including peripheral blood lymphocytes taken from donors of the cell lines for which the antibody is strongly positive (Marti). The antibody precipitates a molecule with a two chain structure which is similar in molecular weight (25,000 and 30,000) to DR antigens, but different in several other respects including the distribution on cells. Most cell lines screened are positive for this antigen and no normal tissue thus far tested has been positive. The antibody is negative for the major antigens of the EB virus and has been shown to be present on cells from a number of individuals with lymphoproliferative diseases such as chronic lymphocytic leukemia and Burkitts lymphoma.

Immunoregulation of T Lymphocytes. Cloned T cell lines and T cell hybridomas with specificity for ovalbumin (Folks) and beef insulin (Schroer) have been produced and they and their products tested for specificity by proliferation, radiobinding, and helper and suppressor assays. For the insulin system, T cell hybridoma antibodies are being used in a variety of studies including some of clinical interest in which human insulin may be detected in the presence of insulin of other species. Idiotypic reagents will be prepared against the B cell hybridoma antibodies and these will be tested for reactivity with T cells that proliferate in the presence of the appropriate insulin sample.

Cellular Immunity to Viruses. Techniques to measure cell mediated immunity in chimpanzees which have been immunized with viral antigens are being studied (Folks, Sell). The goal of this study is to determine the cellular immune responsiveness of chimpanzee to hepatitis virus. At present, control studies using the well characterized vaccinia virus are underway. In these studies MIF assays have shown that positive reactivity can be observed as early as seven days after vaccination. Natural killer functions and other parameters of cellular immunity are also being measured.

Honors and Awards

Dr. Kindt was chosen to serve on the immunology delegation to the Peoples's Republic of China and during his visit there presented seminars on laboratory work in Peking and Shanghai. In addition he chaired the American portion of a joint Chinese-American symposium entitled "Frontiers in Medical Genetics", which was held in Toronto, Ontario in January, 1981. Dr. Kindt was invited as a plenary speaker to the meeting on Protides of the Biological Fluids held in Belgium in May, 1981, and in addition served as chairman of one of the sessions at this meeting. Seminars on lab work were presented in Paris, Marseilles, Geneva, and Basel, Switzerland. He and Dr. John Sogn were invited to teach part of a course in techniques for preparation of hybridomas held at Catholic University this summer. Dr. Kindt has been appointed as Section Editor for Immunochemistry in the Journal of Immunology, and continues to serve on the editorial boards of Current Topics in Molecular Immunology, Journal of Experimental Medicine, Proceedings for the Society of Experimental Biology and Medicine, and Immunology. Dr. Kindt served as Block Chairman for American Association of Immunologists meeting held in the Spring, and at that time was appointed Chairman of the AAI Program Committee, with responsibility for planning the 1982 Meeting of the Society. Dr. Kindt has been appointed adjunct Professor of Microbiology and Pediatrics at Georgetown University School of Medicine and Dentistry.

Dr. John Coligan was invited to present work from his section at the FASEB meeting in Atlanta at a mini-symposium, presented laboratory work in Brussels, Belgium, at the Protides of the Biological Fluids Meeting, and presented a plenary talk at the Symposium on the Clinical Significance of Alien Histocompatibility Antigens, which was held in Wisconsin in the Fall. In addition, Dr. Coligan has been invited to write several review articles concerning his work on the structure of membrane antigens.

Dr. Joyce Schroer was an invited speaker at a symposium on Basic and Clinical Aspects of Immunity to Insulin which was held in Konstanz, West Germany, and was an invited speaker at the NIAMDD Conference on Monoclonal Antibodies and Endocrine Research. Laboratory data were presented at the FASEB meeting at Atlanta by Drs. Sogn, Kimball, Kuo; Dr. Gates presented his data at a mini-symposium at that meeting. LIG scientists were represented at the conference on Idiotypy, held at Salt Lake City this Spring, by Drs. Folks, Jackson, and Dreher, all of whom presented talks or posters at this meeting.

Administrative Changes

Within the past year, Dr. Edward S. Kimball and Dr. F.T. Gates, III left the Laboratory of Immunogenetics to accept other positions. Both had been with LIG since its beginning in 1977. Dr. Gates has gone to the Bureau of Biologics where he will work with Dr. Darrell Liu, and Dr. Kimball has gone to the National Cancer Institute facility at Frederick, where he will work in the Biological Modifiers program. Dr. J. Michael Wilkinson, who has spent two years with the laboratory as an Expert, will return to his permanant position at the University of Birmingham, England, in September.

Within the last month, two Senior Scientists have joined the laboratory and have begun programs which will represent a real expansion of the research potential in LIG. Dr. John Sachs from the University of London, has begun a program on the HLA-D locus, and Dr. Edward E. Max, from the National Institute of Child Health and Development, has begun a program on molecular biological studies of immunoglobulin and histocompatibility genes. Scientists coming to LIG through the Fogarty Center on the Visiting Fellow Program for the year include Dr. Laurent Emorine, from the University of Paris, France, Dr. Li, Xuan-Mao from the Immunology Institute, Shanghai and Dr. Rose Marie van Schravendijk, from Oxford University, England. Dr. Eric Lillehoj will join the laboratory as a Guest Worker with a fellowship from the American Cancer Society, and Dr. Richard Goldsby will join the lab as a Guest worker on sabbatical from his position as Professor of Chemistry at the University of Maryland. Dr. Joyce Schroer joined the Immunobiology Section as an Expert, coming from the Laboratory of Microbial Immunity in our Institute. Dr. Mark Tykocinski from New York University has joined the lab as a medical staff fellow.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00166-04 LIG

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Chemical Characterization of Human and Rabbit Histocompatibility Antigens.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Thomas J. Kindt	Chief, LIG	LIG NIAID
Others:	Dr. J. Michael Wilkinson	Expert	LIG NIAID
	Dr. Claudia Quijano	Visiting Fellow	LIG NIAID
	Dr. John Sachs	Visiting Scientist	LIG NIAID
	Dr. Mei-Chang Kuo	Guest Worker	LIG NIAID
	Dr. John E. Coligan	Res. Microbiologist	LIG NIAID
	Dr. Gerald Marti	C.O.	LIG NIAID
	Dr. Mark Tykocinski	Med. Staff Fellow	LIG NIAID

COOPERATING UNITS (if any)

W. Biddison, NCI, NIH
J.D. Capra, UTHS, Dallas, TX
A. Johnson, Georgetown Univ.

LAB/BRANCH

Laboratory of Immunogenetics

SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.6	2.6	1.

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on molecules encoded at the major histocompatibility complex of the rabbit (RLA) have used monoclonal antibodies (MCAb) prepared against fractions of a rabbit T cell. A 42,000 dalton molecule free of beta-2-microglobulin (B_2-m) was shown by NH_2-terminal sequence analysis and 2-D gel electrophoresis to be identical to RLA molecules isolated by affinity for anti-B_2-m. In spite of apparent identity, the 42,000 dalton molecule associated with B_2-m and that recognized by the MCAb comprise serologically distinct entities. Immunofluorescent studies indicate that the chains reactive with the MCAb are located in the cell cytoplasm.

Studies on human histocompatibility antigens have begun with development of radioimmune assays for for quantitation of HLA-A, -B, -C, and -DR antigens. These assays have been used to determine optimum procedures for isolation of HLA molecules from lymphoid cell lines. The HLA-A3 antigen is being studied in detail to determine if multiple structural forms of this molecule correspond to differences observed in recognition of A3 cells by cytolytic T cells.

Rabbit Histocompatibility Antigens

Little is known concerning the structure of antigens encoded at the rabbit major histocompatibility complex. Analogues of Class I and Class II antigens have been described, but the number of loci involved in their synthesis is not known, and only limited data are available on the primary structure of the Class I antigens. In previous studies the Class I RLA molecules were isolated by affinity chromatography on a column of anti-beta-2-microglobulin (B_2-m). Amino-terminal sequence analysis of the component obtained by this procedure revealed a unique amino acid sequence which showed a high degree of homology to human HLA and murine Class I molecules. The fact that a single amino acid sequence was obtained was unexpected, because a number of molecules are found to be associated with B_2-m in other species.

The structure of rabbit Class I antigens is being reexamined using monoclonal antibodies prepared against glycoprotein fractions of a rabbit T cell line. Two monoclonal antibodies were shown to bind to glycoproteins with molecular weights of 42,000 daltons. These molecules have been shown to be identical by two-dimensional electrophoresis and by amino-terminal sequence to the Class I RLA molecule isolated with anti-B_2-m. It was further shown that the RLA isolated in either fashion is a single species or at most, two molecules, based on 2-D electrophoretic analysis.

The monoclonal antibodies precipitate a 42,000 molecular weight molecule from the RL-5 extracts; however, no reactivity was found with radiolabeled extracts from spleen cells of outbred rabbits. Anti-B_2-m, on the other hand, precipitates 42,000 molecular weight molecules from the normal spleen extracts and these were shown to be heterogeneous by 2-D gel analysis containing 5 or 6 spots. Experiments are underway to determine whether this difference in ability to precipitate molecules is caused by the fact that the MCAb recognize a genetically determined polymorphism in the RLA molecule. Alternatively, it may have to do with a conformational difference caused by the presence or absence of B_2-m in the molecules.

Although anti-B_2-m gives strong positive fluorescence for live cells, the MCAb gave negative results both by visual fluorescence and in the FACS. However, when fixed RL-5 cell preparations were used, strong cytoplasmic staining with MCAb was obtained. This finding argues that the molecule recognized is present in intracellular pools, but is not exported to the cell membrane. Fixed cell preparations from rabbits with a variety of genetic backgrounds will be tested to determine if this positivity is generally true or if there is a genetic discrimination connected with this phenomenon.

Studies on Human Histocompatibility Antigens.

We have recently obtained monoclonal antibodies directed against framework HLA-A,-B,-C determinants and have used the monoclonal antibody in an assay designed to follow isolation of the -A,-B,-C molecules. The assay utilizes radiolabeled antibody and target cells which are fixed with glutaraldehyde.

These fixed cells are no longer succeptible to lysis by NP40, and therefore the extracts used in isolation may be used in the assay as inhibitor. By this method, it has been possible to devise procedures for the isolation of the -A,-B,-C antigens and to quantitatively determine yields at each step of the procedure.

Initial experiments will involve isolation of the HLA antigen A3 from several different cell lines and these will be compared by various structural techniques such as peptide mapping and 2-D electrophoresis in order to determine whether differences in viral restriction patterns observed for these cells can be correlated to differences in primary structure.

In parallel with the isolation of the HLA-A,-B, and -C antigens, studies on the isolation of the HLA-DR antigens are also underway. A number of monoclonal antibodies directed against DR determinants have been obtained from Dr. J.D. Capra and these were tested for their binding to a number of cell lines with differences in their DR regions. Those antibodies with strong binding for a given cell line are used in assays similar to the one above to follow isolation of that antigen through chromatography on lectin columns and through various other chromatographic procedures. It has been found that use of the lectin Ricinus communatis is particularly well suited for the isolation of human HLA-DR antigens.

Our studies on HLA-DR antigens are aimed at isolation of amounts of these antigens in sufficient quantity for use in various functional assays. In addition, structural comparisons will be carried out for DR antigens isolated from individuals with HLA-D identity and those with HLA-D differences. Informative members of a family in which a crossover between the D and DR loci has taken place are available for a study, and cell lines have been derived from peripheral blood from these individuals. In some instances, structural studies will be carried out in collaboration with Dr. J.D. Capra, University of Texas, Dallas.

Appendix

Contract No. N000-14C-79-0886 - Histocompatibility Typing and Infectious Disease.

The research described under human histocompatibility antigens in this project is further supported by contract which is located at Georgetown University under the supervision of Dr. Armead Johnson. This contract provides typing for HLA-A,-B,-C,-D, and -DR antigens. In addition, monoclonal antibodies prepared by LIG are tested for their HLA specificity under this contract.

Publications:

Kindt, T.J.,: A common evolutionary origin for Class I molecules of the MHC and Tla region. Immunol. Today, in press.

Kindt, T.J., Coligan, J.E., Gates, F.T., III, Kimball, E.S., Maloy, W.L., and Wilkinson, J.M.: Beta-2 microglobulin and its association with histocompatibility antigens of the mouse and rabbit. In Peeters, H. (Ed.): Protides of the Biological Fluids, London, Pergamon Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00167-04 LIG

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mass Spectrometry and Sequence Analysis of Polypeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Henry C. Krutzsch	Expert	LIG	NIAID
Others:	Dr. Thomas J. Kindt	Chief, LIG	LIG	NIAID

COOPERATING UNITS (if any)

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NCI, NIH	(Dr. Stuart Rudikoff)
NIMH, St. Elizabeths	(Dr. Shu Yang)

LAB/BRANCH

Laboratory of Immunogenetics

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Immunogenetics Research Section

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NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.1	1.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In immunogenetic studies, rapid and sensitive techniques for protein amino acid sequence analysis are needed. Methodologies involving mass spectrometry (MS) are a promising means to this end. A versatile and sensitive dipeptidyl peptidase approach using gas chromatography-mass spectroscopy (DP/GC-MS) has been developed that allows sequences of picomolar amounts of polypeptides to be determined from the peptide N- or C-terminus. Computer programs further enhance the procedure by simplifying data handling.

The DP/GC-MS method has been utilized to determine partial C-terminal sequences for peptides derived from antibodies and from viruses. Using this information and incomplete sequence data from conventional N-terminal methods, final structural determinations were made. The DP/GC-MS method is currently being tested in studies on the structure of a 225 residue cyanogen bromide fragment from rabbit antibody heavy chain and the variable region of mouse hybridoma antibody heavy chains.

PHS-6040
(Rev. 2-81)

Mass Spectrometry and Sequence Analysis of Polypeptides

Mass spectrometry will be utilized for structural analysis of peptides and proteins. A major objective of this program is to develop and apply the rapid and sensitive DP/GC-MS method to molecules of immunologic interest, such as immunoglobulins, membrane antigens and receptors. Another objective is to increase the sensitivity and scope of the method, such that the method can approach the levels obtainable by radiochemical methods. This will include devising improved stragegies for isolation and purification of small amounts of peptide, such as by HPLC.

Polypeptides to be analyzed by the DP/GC-MS method are digested into dipeptide fragments, either from the N-terminus with dipeptidyl aminopeptidase (DAP), or from the C-terminus with dipeptidyl carboxypeptidase (DCP). The dipeptides are then identified by GC-MS as their trimethylsilyl (TMS) derivatives. Two ions are necessary for mass spectrometric identification: the sequence-determining ion resulting from loss of the C-terminal residue and the N-terminal residue's carbonyl group, and the molecular weight-determining ion, resulting from loss of a silyl methyl group from the parent molecule. The polypeptide is then modified by addition or subtraction of one residue at the N- or C-terminus, respectively, and again digested into dipeptides. Combination of this set of dipeptides and those from the unmodified polypeptide allows reconstitution of the polypeptide sequence.

Application of E. coli DCP for C-terminal peptide digestions in place of the previously utilized Angiotensin Convering Enzyme (ACE) was studied. The E. coli DCP enzyme showed activity on a number of polypeptides ranging in length from 4 to about 110 residues. This enzyme proved to be better for degrading larger polypeptides than was ACE. For example, the insulin B chain, previously degraded with difficulty, is now readily converted into dipeptides with E. coli DCP. Whereas ACE converts tripeptides, the E. coli DCP does not act on these species; therefore digestions using both DCP and DAP enzymes were also done. Large scale quantities of DCP and DAP enzymes were prepared in collaboration with a contractor (Albert Chu, E-Y Labs, San Mateo, CA) who has since built up sufficient stock that they are all now commercially available. The scope of digestions from the DCP enzymes now on hand is similar to that possessed by the DAP enzymes used in this method.

The packed GC column was substituted by a fused silica injector and capillary GC column. The capillary column has proven to be far superior to the packed column, currently allowing identification of as low as 5 pmoles of dipeptide. Dipeptides containing Arg, previously undetectable in the GC mode, were also identifiable, due to conversion to their Orn analogs in the fused silica injector. To take maximum advantage of the capillary GC column, digestions have been further scaled down in size, and only one nanomole of polypeptide is typically used, with 50 pmoles of the trimethylsilylated digestion mixture being injected into the GC-MS.

Sixteen polypeptide samples from Ig chains and viral coat protein, with chain legnths ranging from 22 to 110 residues were analyzed using the DCP method to elucidate their C-terminal sequences. Approximately one nmole of each

[illegible] BETWEEN BLUE OUTLINE [illegible]
[illegible] BE [illegible]LATED UP TO 2 OR 3 [illegible]

polypeptide was used, and the identified dipeptides were aligned by homology to previously determined sequences. The information was then combined with sequence data previously obtained through conventional N-terminal sequencing techniques to yield the primary structures of these polypeptides. This aspect of the method was also used to confirm that the met-enkephalin moiety was at the C-terminus of a50 and a90 residue polypeptide isolated from bovine adrenal glands.

The application of the DP/GC-MS method to the sequence anlaysis of the blocked N-terminal 225 residue cyanogen bromide fragment from a homogeneous a3 allotype rabbit IgG heavy chain is continuing. Becuase the fragments resulting from restricted tryptic digestion (Arg only) are also blocked, the DCP aspect of the DP/GC-MS method has proven to be useful. While ACE gave rather low yields of dipeptides following digestion of the larger fragments (30-80 residues), utilization of the E. coli DCP enzyme, in preliminary studies, has allowed efficient digestion of these molecules and their carboxypeptidase B shortened analogs. Work is now in progress to repeat these digestions using peptide samples further purified to remove material interfering with the GC-MS analysis ofthe digestions. In addition, the larger peptides will be cleaved to smaller peptides to allow their complete sequence analysis to be carried out by this method. Separation of these smaller peptides will be accomplished by HPLC. Structural studies, employing the DP/GC-MS technique, have also begun with mouse hybridoma antibodies.

Work is also in progress on the use of a weak anion exchange HPLC column for the separation of peptides. From the investigations carried out to date, the yields of the peptides eluted form this column are in the 80-90% range, and resolution is excellent. A variety of polypeptides have been included in these studies. Preliminary results indicate that this column will be a valuable complement to the reverse phase columns that are normally used for this purpose.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00168-04 LIG

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure of Rabbit Immunoglobulin and Antibody Heavy and Light Chains

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Thomas J. Kindt	Chief	LIG NIAID

COOPERATING UNITS (if any)

None

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Laboratory of Immunogenetics

SECTION
Immunogenetics Research Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been inactive in the past year.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00169-04 LIG

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Primary Structural Analysis of Murine Transplantation Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	2.3	2.7

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES. ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genes in the mouse H-2 major histocompatibility complex (MHC) determine a diversity of functions associated with immune recognition and reaction. Several genes in this region e.g. H-2K, D, L and R encode antigens involved in allograft rejection and T-lymphocyte responses. The purpose of this work is to determine the primary structure of these molecules in order to better understand their function and mechanism of action. The determination of the 346 amino acid residue, H-2K^b membrane-bound molecule has been completed. For comparative purposes, major portions of the primary structure of four other H-2 molecules (D^b,K^d,D^d and L^d) have been determined. These studies indicate that most of the amino acid sequence differences among these molecules are localized to discrete regions. In addition, H-2 molecules have been found to vary in length and in the number of carbohydrate prosthetic groups that they possess. Sequential immunoprecipitation studies have revealed the presence of a previously unidentified D-region molecule in the d and q haplotypes (H-2R). Elucidation of the complete primary structure of murine B_2m has revealed a genetic dimorphism: an Ala-Asp interchange occurring at position 85.

PHS-6040
(Rev. 2-81)

Primary Structural Analyses of Murine Transplantation Antigens.

A major objective of this research is to gain a better understanding of the molecular structure of membrane molecules of lymphoid cells with emphasis on those playing prominent functional roles. Knowledge of the structures of these molecules will hopefully provide insight into how these molecules function, i.e. recognize extracellular signals, transmit these signals across the cell membrane, and how they maintain their orientation in the membrane. In addition, these structural studies should define evolutionary relationships among these molecules providing clues to their origins.

Structure of H-2K^b

The determination of the complete amino acid sequence of the H-2K^b molecule has been completed using radiochemical methods. This includes the 346 residue heavy chain as well as its associated 99 residue light chain, B_2-m. The heavy chain consists of three functional segments: the extracellular segment (residues 1-281), the membrane-bound segment (residues 282-308) and the cytoplasmic segment (residues 309-346).

The extracellular segment of the molecule can be readily divided into three regions (domains) of 90 residues. The division of the extracellular portion of the molecule into such regions analogous to Ig-like domains is prompted by primary structural homologies of H-2 and B_2-m to IgG constant region domains. This division is supported by the location of the two points where the molecule is susceptible to cleavage by the proteolytic enzyme, papain. Susceptibility at these points (approximately at residues 180 and 281) suggests an interruption of a domain-like structure of a globular protein. The domains are designated N (residues 1-90) for the NH_2-terminal portion and C1 (residues 91-180) and C2 (residues 181-270) for Cys containing segments.

A major feature of the molecule is the ease with which the intact extracellular region can be cleaved from the cell surface with papain. This site of papain cleavage (residue 281) approximately coincides with the beginning of a stretch of uncharged and hydrophobic amino acids of approximately 25 residues in length. This stretch of nonpolar amino acids is thought to be a membrane-binding subregion. (Twenty-five residues in the conformation of an -helix could span 35A, the approximate thickness of the hydrocarbon core of a lipid bilayer.) At the carboxy-terminal end of the membrane-binding region is a cluster of basic amino acids (positions 308 and 310-312) that may serve to anchor the H-2 molecule in the membrane through interaction with the negatively charged phosphate groups of the membrane phospholipids. Glycophorin, membrane-bound IgM, and HLA have similar clusters of basic residues at the end of the membrane-binding portion of the molecules. That portion of the molecule beginning at the inner face of the membrane and stretching to the C terminus is apparently located inside the cell and possibly functions in the communication of signals to the cell interior following interactions of the exposed extracellular region of the molecule.

Studies of other Murine Class I Antigens.

Although H-2K^b is the only murine histocompatibility antigen for which a complete primary structure is known, extensive areas of the primary structure of several other H-2 molecules have been determined in our laboratory and nearly complete primary structure have been reported for the human HLA-B7 and -A2 molecules, as well as for a pool of human HLA antigens. Structural features apparently conserved by all of these molecules include the two intrachain disulfide loops which span positions 101-164 and 203-259 and the carbohydrate moiety which is linked to an Asn residue at position 86. In addition, all H-2 molecules, but none of the HLA molecules studied, possess a second carbohydrate moiety attached to the Asn at position 176. It recently has been observed that certain H-2 molecules, e.g. H-2D^b and H-2K^d, possess a third carbohydrate moiety. The attachment of this third carbohydrate moiety has been localized to position 256 in H-2D^b.

The availability of the amino acid sequence of the NH_2-terminal 100 residues from four H-2 and two HLA molecules, as well as for a pool of HLA molecules, permits extensive comparisons to be made for this portion of the molecules. These comparisons reveal that a high degree of primary structural homology exists among these molecules. The homology among the H-2 molecules in this region ranges from a low of 76% (D^d vs K^d) to a high of 87% (D^d vs K^b). Comparison between H-2 and HLA molecules yields about 70% homology in all cases. Further examination of the NH_2-terminal sequence data indicates that 63 residues are common to all H-2 molecules. Of these 63 residues, 54 (or 86%) are also common to HLA. Even more remarkable is the fact that at 86 of the 100 positions at least one H-2 and one HLA molecule share a common amino acid. In addition, only 1 of the 14 positions where common amino acids are not shared requires a multiple base change in the DNA-coding sequences to account for the substitution.

Examination of the NH_2-terminal 100 residues of the H-2 molecules reveals that there are 37 positions where at least two different amino acid residues occur in the sequence. The majority of these differences are nonrandomly distributed. Small clusters of differences are evident at positions 18-24, 30-32, 41-45 and 95-100, and a large concentration of differences is found between positions 61 and 83. These clusters of differences are interspersed with stretches of conserved amino acid residues. All of these regions represent areas of diversity between the murine and human molecules; however, only the large cluster of differences (61-83) represents an area of diversity when only the human molecules are compared. It is tempting to speculate that the amino acids interchanges observed at positions 61-83 represent an important region of diversity among these alloantigens. In support of this possibility, the homology in this region is strikingly lower than the overall NH_2-terminal homology. Only four of the 23 positions (17%) are common to the six molecules whereas 50 of the remaining 77 residues (65%) are identical. Likewise, only 10 residues (43%) are shared among the four H-2 molecules in region 61-83 whereas 53 (69%) are shared for the remaining 77 residues. Furthermore, 7 of the 10 amino acid conversions which require two base changes at the nucleic acid level for interconversion occur in this region.

The extensive sequence data available for H-2K^b and HLA-B7 allow comparisons to be made over the entire length of these molecules. Several points concerning this comparison can be made. Although the overall homology between these molecules is 71% for positions 1-281 (extracellular portion), the homology for those after position 281 drops to 34%. In contrast, the homology between the human molecules (87%) is similar to that observed for the extracellular region of the molecule. In addition to the striking degree of sequence difference between the murine and human molecules, the K^b molecule is 10 residues longer than the human molecules. In order to achieve maximum alignment of the K^b and B7 molecules, a deletion must be placed at position 307 of the B7 molecule.

That there is significantly less homology between K^b and K^d (presumed alleles) than between K^b and D^b is intriguing. This apparent lack of "K-ness" or D-ness" which agrees with previous conclusions from peptide mapping data is difficult to reconcile with the concept that the multiple histocompatibility gene loci arose by gene duplication in a common ancestor. In such a model, divergence of the allelic genes would arise through mutations occurring after speciation. The present data suggest that a gene duplication event occurred subsequent to speciation. This is supported by the large number of species-associated residues which occur throughout the length of the molecules. (Species-associated residues characterize the gene-products from a gene family of one species as different from those of another species.)

Plans for next year include further studies of the primary structure of H-2 K^d, D^d and D^b, molecules for which extensive information is already available, and of H-2 L^d and R^d, molecules for which little (L^d) or no (R^d) primary structural data is available. In conjunction with these studies, we hope to undertake peptide mapping studies to determine the relationship of the H-2D^q, L^q and R^q molecules to the L^d and R^d molecules.

Structure of Murine B_2-m.

The complete primary structure of murine B_2-m has been determined. This study revealed that murine B_2-m is dimorphic in that B_2-m isolated from EL4.BU tumor cells (C57BL/6) have an alanine residue at positions 85 whereas B_2-m isolated from C14 tumor cells (BALB/c) has aspartic acid at this position. Genetic analysis of the B_2-m polymorphism by other laboratories has revealed that it is linked to the H-3 and Ly-4 loci on chromosome 2.

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Kimball, E.S., Maloy, W.M., and Coligan, J.E.: Evidence for three carbohydrate prosthetic groups on mouse histocompatibility antigens H-$2K^d$ and H-$2D^b$. Mol. Immunol. 18: 667-680, 1981.

Publications (cont.)

Uehara, H., Coligan, J.E., and Nathenson, S.G.: Isolation and sequence analysis of the intramembranous hydrophobic segment of the $H-2K^b$ murine histocompatibility antigen. Biochemistry, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00170-04 LIG

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structural Studies on Precursors to Immunoglobulin Molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Others:	Dr. Frederick T. Gates	Staff Fellow	LIG	NIAID

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0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been inactive in the past year; a publication of work completed in the previous year has appear.

Publications

Singer, H.H., Gates, F.T., Kindt, T.J., and Williamson, A.R.: Partial amino acid sequence of precursors of blocked heavy and light chains from a C3H mouse myeloma: Identification of a new V_H subgroup. Eur. J. Immunol. 10: 346-351, 1980.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00171-04 LIG

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Studies on Rabbit Immunoglobulins and other Serum Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Dr. Mei-chang Kuo	Guest Worker	LIG NIAID
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Improved techniques for stabilization of rabbit-mouse hybridomas have allowed the preparation of a number of new cell lines. Although all of the cell lines which were prepared in the past secrete either an immunoglobulin heavy or light chain, but not both, two of the most recently prepared cell lines appear to secrete both heavy and light chains from the rabbit. Characterization of these and other hybridoma products is well underway. These studies have revealed several unusual serological and structural characteristics of the secreted products. In the course of the fusion experiment directed toward preparation of rabbit-mouse hybridomas it was observed that rabbit spleen cells, when in culture, give rise to a large cell which has a long life in culture. Characterization of these cells indicated that they were nonadherent macrophages. In collaborative studies with scientists at the Institut Pasteur in Paris, structural characterization is being undertaken for allotypes which are found only in wild rabbit populations. The serologic characterization of the light chains indicates that they have allotypic markers which are similar to but not identical with those from the domestic rabbit population.

Preparation of Rabbit-Mouse Hybrids

Improved procedures have expedited the preparation and maintainence of stable rabbit-mouse hybridomas that secrete rabbit immunoglobulin (Ig) chains. A major problem in screening for rabbit-mouse hybridomas was interference by rabbit spleen cells which secrete Ig for a number of days following the fusion procedure. Interference by such endogenous Ig secretion has been minimized by isolation of the hybrid cells on a fetal calf serum gradient three days after fusion. It has been observed that rabbit-mouse hybrids may require as many as five cloning steps in order to obtain a reasonable level of cell line stability as indicated by a 90% frequency of positive cells upon cloning. Following stabilization, rabbit-mouse hybridoma lines are stable in culture for up to two months, after which they may require restart from frozen cultures or recloning. Use of highly specific assays has allowed rapid characterization of the hybrid products, thus facilitating the multiple clonings necessary to maintain these lines. Growth and secretion characteristics have been determined for a number of the stable rabbit-mouse hybrid cell lines. Both H chain and L chain producing hybrids have been propagated as ascites in BALB/c nu/nu mice. The H chain producers yield no undegraded product in ascites, whereas L chains undergo variable amounts of proteolytic degradation. An H chain producing hybrid has been propagated successfully in normal BALB/c mice after adaptation by two passages in nude mice.

Ig Products of Rabbit-Mouse Hybridomas

Serologic and primary structural analyses of Ig chains secreted by rabbit-mouse hybridomas have shown that these hybrid cells produce H or L chains identical to those isolated from rabbit sera. Two of the cell lines secrete rabbit H chains which express a full complement of variable and constant region allotypes. However, these apparently normal rabbit H chains are secreted in a complex with a molecular weight of 130,000 and serologic studies indicate this complex contains a covalently linked mouse kappa light chain. Other cell lines were shown to produce rabbit light chains with molecular weights of 23,000 and 25,000 respectively. Serologic analyses indicated that the allotypes of these chains were equivalent to those expressed by normal rabbit Ig molecules. Partial amino acids sequence data verified the rabbit nature of these L chains and showed them to be different from the mouse L chains expected to be secreted by the parent myeloma cell line.

Three hybridomas of unusual interest have been recently identified. One secretes a product expressing the V_H allotype a1 and the C_K allotype b4, another a1 and b6, and a third secretes a product expressing no allotypes. The first is likely to be an IgG molecule but the second lacks some of the characteristics of IgG and may be IgM. The third may be the product of one of the nonallelic V_H genes of the x or y series. Studies using intrinsically labeled, affinity purified hybridoma products are under way to answer these questions.

Characterization of Rabbit Splenic Nondherent Macrophages

During the preparation of rabbit-mouse hybridomas, an unusual degree of cellular proliferation and a high level of rabbit Ig secretion in culture was noted. The proliferation and Ig secretion, which were subsequently found to occur also in normal rabbit spleen cell cultures, obscure the early proliferation of hybrid cells and make it difficult to detect rabbit Ig chain secretion by the interspecies hybridomas. The proliferation appears to be dependent on the use of a relatively high level (10%) of fetal calf serum because it was not seen when spleen cells were cultured using either horse serum or autologous serum in a variety of media. Identical results were obtained with 6 different lots of fetal calf serum from 4 different suppliers. The peak of the proliferative response occurs at 7 to 10 days in culture, after which the lymphoblast population dies rapidly, leaving a large, granular, nonadherent cell as the predominant component in the culture. These cells do not appear to divide further but persist in very gradually diminishing numbers for many months. This long-lived cell has been characterized as a nonadherent macrophage by its morphology, intense esterase staining, expression of an Fc receptor for rabbit IgG, phagocytosis of latex particles and opsonized sheep erythrocytes and mediation of antibody-dependent cell-mediated cytotoxicity on chicken erythrocytes.

Allotypes of L Chains Isolated from Wild Rabbits

Dr. Pierre-Andre Cazenave and his co-workers at the Institut Pasteur have reported the presence of previously undetected allotypic specificities in the wild rabbit population on the island of Zembra. By cross breeding experiments they have introduced the genes for these allotypes into their domestic rabbit population and have given us serum from these rabbits for characterization of the structural aspects of these L chains. Standard structural techniques will be used to determine whether these L chains, which have crossreactivity but no identity to allotypes of L chains from domestic rabbits, have structures similar to those reported for the rabbit domestic allotypes of the b series.

Hamster Female Protein

Collaborative studies on hamster female protein with Dr. John Coe are continuing. Preliminary steps have been taken in the structural study of this unique serum protein.

Publications:

Coe, J.E., Margossian, S.S., Slayer, H.S., and Sogn, J.A.: Hamster female protein. A new pentraxin structurally and functionally similar to C-reactive protein and amyloid P component. J. Exp. Med. 153: 977-911, 1981.

Yarmush, M.L., Gates, F.T., III, Dreher, K., and Kindt, T.J.: Serologic and structural characterization of immunoglobulin chains secreted by rabbit-mouse hybridomas. J. Immunol. 126: 2240-2244, 1981.

Sogn, J.A., and Jackson, S.: Long-live nonadherent rabbit macrophages obtained from spleen cell sultures. Submitted for publication.

Capra, J.D., and Kindt, T.J.: Antibody diversity: Something for everyone. Scand. J. Immunol. 13: 205-209, 1981.

Capra, J.D., and Kindt, T.J.: One from many: immunoglobulin V regions are the products of interacting genes. In The Strategies of Immune Regulation, New York, Academic Press, 1980, pp. 233-235.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00172-04 LIG

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Carbohydrate and Glycoprotein Antigens of Microbial Cell Walls

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. John Coligan   Research Microbiologist   LIG   NIAID

COOPERATING UNITS (if any)

Dept. of Micro., Univ. of Alabama at Birmingham (Dr. David Pritchard)

LAB/BRANCH
Laboratory of Immunogenetics

SECTION
Membrane Antigen Structure Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS   ☐ (b) HUMAN TISSUES   ☒ (c) NEITHER

☐ (a1) MINORS   ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In previous studies, the sequence of sugars in the repeating unit of the Groups A, A-variant and C streptococcal polysaccharides were determined. In similar studies on the Group B polysaccharide, the composition and nature of the linkages of its component monosaccharides have been analyzed. This carbohydrate contains 1-linked rhamnose, 1,2-linked rhamnose, 1,2,4-linked rhamnose, 1,3-linked galactose, 1,3-linked N-acetylglucosamine and glucitol-phosphate. The linkages of glucitol phosphate are as yet undetermined.

PHS-6040
(Rev. 2-81)

Carbohydrate and Glycoprotein Antigens of Microbial Cell Walls.

Having determined the composition and linkages of the monosaccarides involved in the structure of the streptococcal Group B-specific polysaccharide, we are in the process of determining the structure of the repeating unit of this polysaccharide. In addition, the nature (α or β) of the anomeric linkages in this polysaccharide are being determined by NMR spectroscopy as are the linkages of the Groups A, A-variant and C streptococcal polysaccharides. We had previously determined the structures of the repeating units of the latter polysaccharides except for the nature of the anomeric linkages.

Publications:

Pritchard, D.G., Coligan, J.E., Speed, S.E. and Gray, B.M.: Carbohydrate fingerprints of streptococcal cells. J. Clin. Microbiol. 13: 89-92, 1981.

Pritchard, D.G., Brown, G.B., Gray, B.M. and Coligan, J.E.: Glucitol is present in the group-specific polysaccharide of Group B streptococcus. Current Microbiol. in press.

Pritchard, D.G., Coligan, J.E., and Egan, M.L.: Etude chimque de l'ACE. In l'Antigene Carcino-Embryonnaire, Aspects Medhodologiques et Cliniques, Paris, Edition Medicale Universitaire, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00173-04 LIG

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Nonallelic Expression of Genes Encoding Rabbit Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Thomas J. Kindt	Chief	LIG NIAID
Others:	Dr. John A. Sogn	Chemist	LIG NIAID
	Dr. F.T. Gates, III	Staff Fellow	BOB FDA
	Dr. Susan Jackson	Staff Fellow	LIG NIAID
	Dr. Kevin Dreher	Staff Fellow	LIG NIAID
	Dr. Edward Max	C.O.	LIG NIAID
	Dr. Laurent Emorine	Visiting Fellow	LIG NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Immunogenetics

SECTION
Immunogenetics Research Section

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NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.6	2.75	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Descriptions of genetically unexpected immunoglobulins (latent allotypes) in several species suggest that immunoglobulin gene complexes contain more genetic information than is normally expressed, a possibility that should require a considerable revision in current concepts of the genetic control of Ig expression. Our studies have emphasized latent allotypes in the rabbit and have attempted to determine conditions for in vivo induction of latent allotypes. In conjunction with these studies an ELISA has been developed. Other studies attempt to determine the molecular biological basis for latent allotypes. Rabbit-mouse hybridoma cells have been used as a source of mRNA of the Ig chain. This is being purified to use in construction of DNA probes for determining the structure of the Ig genome in the rabbit.

Induction of Latent Allotypes

Although there have been extensive studies on serum latent allotypes, there have been virtually no data available on the physiological aspects of latent allotype expression. In the past year, we have attempted to determine what factors control the rapid disappearance and reappearance of latent allotypes. Clearance rates of passively administered IgG of different allotypes were measured in a number of rabbits. Using a paired label technique, it was shown that foreign allotypes were cleared more rapidly than self alloyptes in a few of the rabbits tested, although the rest cleared self and foreign allotypes at identical rates. Rapid clearance of a foreign allotype correlated with a history of expression of that allotype as a latent allotype in the rabbit tested. In other experiments, immunization against anti-allotype antibodies markedly increased serum levels of latent allotypes. The data obtained suggested that an allotypic network, similar to an idiotypic network, may exist in rabbits to activate and/or to remove latent allotypes.

In a continuation of the latter experiments, we have injected rabbits with anti-allotype antibody, and after resting these animals for suitable periods, we have initiated immunization with ovalbumin. These experiements were designed so that we could isolate ovalbumin-specific antibody from affinity columns and determine its allotypic composition. The advantage of this approach is that in assaying affinity-purified antibody for the presence of latent allotype, we should preclude the presence of anti-(anti-allotype) in the sample. The latter antibody would interfere in the ELISA we have developed (Jackson, Sogn and Kindt, submitted for publication) to measure rabbit allotypes in serum samples and culture supernatants.

An ELISA has been adapted for the determination of rabbit allotypes. This assay is particularly useful in the investigation of latent allotypes, because it is capable of detection levels as low as 40 nanograms/ml, and because it is simple, rapid, and inexpensive. It is not subject to interference from anti-allotype antibody, which may be present in the test sample and does not generate radioactive waste as did the RIA previously used for this purpose.

Molecular Biological Studies of Rabbit Allotype Genes.

Laboratory efforts have focused upon the use of rabbit-mouse hybridomas as a source of mRNA for homogeneous rabbit immunoglobulin chains. This mRNA will be enriched with respect to the fraction that encodes the heavy or light chain from the cell line and this enriched fraction will be used to construct cDNA probes that will be used for further molecular biological studies.

In the past year, effort has focused on obtaining large amounts of the rabbit-mouse hybridomas to be used as a source of mRNA. Because these are interspecies hybridomas, propagation in normal BALB/c mice is impossible. Early attempts to propagate these in nude mice have been successful, but yields from ascites tumors in the nude mouse have not been satisfactory. As an alternative procedure, we have grown the rabbit-mouse hybridomas as solid tumors, and this has proved to be the most successful method of propagation. It has furthermore been possible to adapt certain of the rabbit-mouse hybridomas to grow in normal BALB/c mice after several passages in nude mice.

One cell line, 12F2, has been propagated as a solid tumor. mRNA has been isolated and translated in vitro. The material from this translation mixture which reacted with antibody raised against rabbit light chain was isolated and subjected to SDS gel electrophoresis. A band with molecular weight 25,000 was cut out and amino acid sequence analysis was performed on the material radiolabeled in leucine. The sequence obtained indicated that the chain had a precursor with approximate length of 19 residues and that the amino terminal portion of the chain corresponded to that which was isolated from cell culture or from ascites fluid in which 12F2 had been propagated.

In recent experiments the mRNA from 12F2 has been enriched with respect to the light chain mRNA by sucrose density gradient centrifugation. This material will be copied by reverse transcriptase and the resultant cDNA will be cloned in plasmids for further study.

Appendix

Contract N01 AL 02652 - Maintenance of a Rabbit Breeding and Holding Facility.

This contract has objectives and uses methods related to and in support of the above research project. In addition support is provided for LIG projects Z01 AI 00171-04, Genetics Studies on Rabbit Immunoglobulins and other Serum Proteins, and Z01 AI 00166-04, Chemical Characterization of Human and Rabbit Histocompatibility Antigens. Recently the contract has included rabbit holding, injection and bleeding for about 100 rabbits belonging to other (non-LIG) NIAID intramural scientists. The contract is held by Spring Valley Farms.

Publications:

Yarmush, M.L., Sogn, J.A., Kern, P.D. and Kindt, T.J.: Role of immune recognition in latent allotype induction and clearance: Evidence for an allotypic network. J. Exp. Med. 153: 196-206, 1981.

Kindt, T.J., and Yarmush, M.L.: The significance of latent allotypes to regulation of immunoglobulin and histocompatibility gene complexes. In Atassi, M.Z. (Ed.): Critical Reviews in Immunology, CRC Press, Inc., in press, 1981.

Jackson, S. and Mestecky J.: Oral-parenteral immunization leads to the appearance of IgG auto-anti-idiotypic cells in mucosal tissues. Cell. Immunol. 60: 498, 1981.

Jackson, S., Kulhary, R. and Mestecky, J.: Shared idiotypes among anti-albumin antibodies of different species. Scand. J. Immunol., in press, 1981.

Mestecky, J., McGhee, J., Crago, S., Jackson, S., Kilian, M., Kiyono, H., Babb, J., and Michaelek, S.: Molecular-cellular interactions in the secretory IgA response. J. Reticuloendothel. Soc. 28: 45s, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00180-03 LIG

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies Directed Against Cell Surface Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. John A. Sogn	Chemist	LIG NIAID
Others:	Dr. Thomas J. Kindt	Chief	LIG NIAID
	Dr. J. Michael Wilkinson	Expert	LIG NIAID
	Dr. Gerald Marti	C.O.	LIG NIAID

COOPERATING UNITS (if any)

T. Chusid, LMI, NIAID

LAB/BRANCH
Laboratory of Immunogenetics

SECTION
Immunogenetics Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	1.6	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Monoclonal antibodies (MCAb) have been prepared using extracts of the rabbit T cell line RL-5. A total of 28 MCAb selected for positive reactivity with RL-5 and nonreactivity with the human cell line Daudi were tested by immunoprecipitation and surface fluorescence. The molecules precipitated by the MCAb fell into three major groups with molecular weights 120,000, 90,000 and 42,000. Reactivity of the MCAb with subpopulations of rabbit lymphoid cells is being investigated by FACS analysis.

Attempts to prepare monoclonal antibodies directed against polymorphic determinants of HLA antigens resulted in an antibody that reacts with a number of lymphoid cell lines but with no normal cells, including peripheral blood lymphocytes taken from the donors of EBV transformed cell lines for which the antibody is strongly positive. The antibody precipitates a molecule with a two chain structure, the chains having molecular weights of 25,000 and 30,000 respectively.

Antibodies directed against rabbit lymphoid cell populations.

Although the humoral immune system of the rabbit has been studied in some detail, little is known of the rabbit cellular immune system. This lack of knowledge is mainly because there are very few rabbit lymphoid cell lines and there are difficulties in obtaining completely inbred rabbit strains. Attempts to characterize lymphoid T and B cell populations of the rabbit have used immunologic reagents, and a number of heterologous anti-rabbit T cells reagents have been described. A far superior approach to this problem is given by the advent of hybridoma technology. We have used the rabbit cell line RL-5, which is a line with T cell characteristics, and have used fractions from this line to immunize BALB/c mice for the preparation of hybridomas. A total of 28 antibodies with differing reactivities were identified.

Classification of the monoclonal antibodies by their ability to immunoprecipitate radiolabeled molecules from lysates of RL-5 fractionated by passage over lectin columns placed the antibodies in three major categories. The antibodies precipitated molecules of molecular weights of 120,000, 90,000, or 42,000, respectively. At least three different specificities were observed for the molecules with molecular weight 90,000. These differences were based on fractionation of the molecules by lectin chromatography; one group being positive for wheat germ agglutinin, the other for lentil lectin. The antibodies directed against the 42,000 molecular weight molecule appear to be directed against an RL-A Class I molecule which occurs in the cell lysate free of beta-2-microglobulin. The characterization of this molecule is described above (Project 00166-04).

Representative monoclonal antibodies from each group were studied by Flow cytometry on the RL-5 cell line and subsequently on rabbit lymphoid cells from peripheral blood, spleen, thymus, and lymph nodes. Using the monoclonal antibodies in single and double fluroescent studies, it has been ascertained that certain of these are specific for rabbit T cells while others have more general reactivity for rabbit lymphoid cell populations. None of the antibodies gives a strong reaction with human peripheral blood lymphocytes.

The molecules precipitated by the antibodies have been characterized by two-dimensional gel electrophoresis and by sequential immunoprecipitations to determine whether the molecules recognized were identical one to another. In cases where identity was indicated, two color fluorescent experiments were carried out on the Flow cytometer in order to determine whether one antibody blocked binding of the other. The results of these studies are being prepared for publication.

Monoclonal Antibody Directed Against a Human Lymphoid Cell Antigen.

Studies directed toward formation of monoclonal antibodies with reactivity for polymorphic determinants of human HLA antigens resulted in an antibody with reactivity for a cell surface determinant which has not yet been fully characterized. This rather unusual determinant is found on most lymphoid cell lines studied. It is not found, however, on peripheral blood lymphocytes including those from donors of certain of the cell lines for which it has positive reactivity. Precipitation analysis indicates that a molecule with two chains with molecular weights of approximately 30,000 and 25,000 is precipitated. There is a slight lowering of the apparent molecular weights of the two chains when nonreducing conditions are employed in its analysis.

Preliminary experiments suggest that the determinant may be present on T cells following activation with the mitogen PHA. This observation will require further study of the reactivity of the antibody with various different cell lines and with cells from individuals with lymphoproliferative diseases such as CLL. The products precipitated by the cell lines will characterized to determine their similarity to antigens of known structure.

Appendix

Contract N01 AI 92612 - Establishment of a Tissue Culture
Laboratory and Animal Facility.

This contract operates in support of this project, project Z01 AI 00173-04, Nonallelic Expression of Genes Encoding Rabbit Immunoglobulins, and project Z01 AI 00166-04, Chemical Characterization of Human and Rabbit Histocompatibility Antigens. The contract is located at Biotech Research Labs in Rockville, Maryland, and provides facilities for the preparation, maintenance, and propagation of hybridoma cell lines in culture and in BALB/c mice or nude mice as ascites fluid. In addition, this contract has supported project Z01 AI 00191-03, Immunoregulation of T Lymphocytes, by the preparation of T cell growth factor and by propagation of anti-insulin hybridomas prepared by Dr. Schroer.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00191-03 LIG

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunoregulation of T Lymphocytes - The Role of Anti-idiotype and Immune Complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Kenneth W. Sell	Head	LIG NIAID
Others:	Dr. Thomas M. Folks	Staff Fellow	LIG NIAID
	Dr. John A. Sogn	Chemist	LIG NIAID
	Dr. Joyce Schroer	Expert	LIG NIAID
	Dr. Susan Jackson	Staff Fellow	LIG NIAID

COOPERATING UNITS (if any)

J. Rosenzweig, NIAMDD, NIH
L. Claflin, University of Michigan

LAB/BRANCH
Laboratory of Immunogenetics

SECTION
Immunobiology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.45	2.25	2.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

T cell lines and T cell hybridomas for ovalbumin and beef insulin have been produced and tested for specificity by proliferation, radiobinding and helper/suppressor assays. Characterization of the factor(s) released by these cell lines is currently underway. For the insulin system, B cell hybridoma antibodies are being used to prepared idiotypic reagents which will be tested for T cell reactivity. In other studies, cellular immunity to vaccinia virus in the chimpanzee has been studied using natural killer, MIF, and proliferation assays. HLA and DR typing of the chimpanzee is being carried out in order to determine the genetic basis for viral antigen presentation.

PHS-6040
(Rev. 2-81)

Antigen Reactive T Cell Factors

A practical approach to understanding regulation of the immune response is to establish antigen specific T cell lines and clones. Once these cells are established and can be confirmed as specific for the antigen in question, then it may be possible to further characterize the molecules (factors or receptors) contributing to immune regulation.

Our work in the last year has concentrated on the establishement and maintenance of T cell lines derived from murine spleens following antigen innoculation. The antigens utilized have been ovalbumin, human serum albumin and beef insulin. One ovalbumin specific T cell line (021 ST9.4) was found to secrete two proteins (43,000 and 12,000 daltons) which bound to ovalbumin-Sepharose. The line was extremely unstable and unable to be maintained as a specific secretory line. Surface markers found on this cell line by FACS analysis were theta, Ly-1, and possibly Ly-2.

Many other T cell lines have been established, but with no success at antigen binding either directly to the cell surface or via a secreted product. Improved cell specificity as determined by proliferation has been achieved by "resting" blasted T cells with irradiated spleen cells for approximately one week before restimulating with antigen. Cell lines treated in this way have shown helper activity in AFC assays using antigen primed B cells and T cells or supernatants from T cell lines. These same T cell lines, expanded with TCGF, have resulted in suppressor activity in PFC assays.

Since direct specific antigen binding activity is low or nonexistent in these T cell lines, we have begun to fractionate supernatants from these lines and assay by functional criteria (helper or suppressor activity).

T cell lines have been developed from rabbit PBL. This line is specific for Group C streptococcal vaccine. At time of writing it has been maintained in vitro for over 2 months in the presence of autologous irradiated presenting cells with whole vaccine and/or in conditioned media (supernatants from rabbit splenocytes cultured overnight in the presence of Con A). To date, the line has not been cloned. At present, we are attempting to isolate antigen (streptococcal) binding factor(s) from culture supernatants of this line. Furthermore, a similar line from a second rabbit has been initiated. The lines can provide a tool for characterization of rabbit T cell responses.

Mapping Antigenic Determinants on the Insulin Molecule

We have continued our studies of the B cell response to insulin using the technique of somatic cell hybridization. The B cell response to insulin is important because it provides a means to map antigenic determinants on the insulin molecule and the defined anti-insulin antibodies may be used for other experiments such as studies of the biosynthesis of insulin.

Six new anti-insulin hybridomas against human insulin and beef insulin have been prepared. These are superior to the previous previous four hybridomas in that they are the products of fusion with the SP2-0 total nonsecretor cell line and make only the desired anti-insulin antibody (the previous four antibodies made with the NS1 cell line have an irrelevant light chain).

In collaboration with L. Claflin of the University of Michigan Medical School, we have begun to characterize for affinity and specificity seven more anti-beef insulin hybridomas generated at Michigan. Recently, using species variants of insulin to inhibit the binding of hybridoma antibody to ^{125}I-beef insulin, we have determined the exact location of the antigenic determinant recognized by one of the hybridomas, AE 9 D6, made against human insulin. This antibody binds to a 3-dimensional determinant formed around residues 8-10 of the A chain of the insulin molecule. This region of the molecule has been shown to be critical for T cell recognition of beef insulin in the H-2^{b} strain of mice. Thus B cell products, antibodies, and T cells recognize similar determinants on the insulin molecule.

Other Experimental Uses of Anti-Insulin Hybridomas

Drs. J. Rosenzweig and S. Taylor of the Diabetes Branch of NIAMDD have been using some of the anti-insulin hybridoma antibodies to determine the species origin of a given insulin. Dr. Rosenzweig initially demonstrated that rat brain cells in culture make autologous insulin. A monoclonal anti-insulin antibody with specificity for beef insulin was used to show that the insulin present in cell extracts was not coming from fetal calf serum used in the culture. Dr. Taylor is currently using this same antibody and the AE9D6 antibody to evaluate individual patients with possible insulinomas for surreptitious injections of pork or beef insulin.

One final project begun this year involves attempting to make autologous anti-insulin hybridoma antibodies by immunizing mice with rat insulin (mouse and rat insulins have the same amino acid sequence). In our previous work with the four initial anti-beef insulin hybridomas, one of these antibodies bound rat insulin with high affinity, only 10-fold less than the affinity for beef insulin, suggesting that autologous antibodies are made.

Cellular Immunity to Viruses

A second project which utilizes assay techniques for cell-mediated immunity is a study involving the responsiveness of chimpanzees to viral antigens. Ultimately, the goal is to study the cellular immune responses of the chimpanzee to heptitis virus. Before we can begin this, however, it is necessary to establish the CMI parameters of the chimpanzee to a well characterized virus. The chosen virus is vaccinia.

Our results have indicated a significant proliferative response of chimpanzee PBL to vaccinia at 21 days post vaccination. MIF results have shown that positive reactivity can be observed as early as 7 days. Natural killer function was greatly enhanced with 21 and 28 day cells following restimulation with virus in culture for 7 days (manuscript in progress).

Studies are also underway to establish chimp T cell lines responsive to vaccinia, the goal being to determine if human PBL can serve as presenting cells for the chimpanzee. DR typing of both human and chimp cells are being carried out to establish the relationships between viral antigen presentation and proliferation. Screening of normal human donors for proliferative responses to vaccinia and NK activity have been undertaken as well.

In addition to CMI studies in the chimpanzee, a project in collaboration with Dr. John Sachs to correllate the human lymphocyte proliferative response to vaccinia virus with HLA has also begun.

Publications:

Burger, R., Clement, L., Schroer, J., Chiba, J., and Shevach, E.M.: Monoclonal antibodies to guinea pig Ia antigens I. Production, serological, and immunochemical characterization. J. Immunol. 126: 32-37, 1981.

Hunter, K.W., Folks, T.M., Sayles, P.C., and Strickland, T.G.: Early enhancement followed by suppression of natural killer activity during murine malarial infections. Immunol. Lett. 2: 209-212, 1981.

Schroer, J.A.: Hybridoma antibody recognition of the insulin molecule. In Fellows, R.E. (Ed.): Monoclonal Antibodies in Endocrine Research. New York, Raven Press, in press.

Schroer, J.: Mouse hybridoma antibody recognition of the insulin molecule. In Keck, K., and Erb, P. (Eds.): Basic and Clinical Aspects of Immunity to Insulin. Berlin, N.Y., De Gruyter & Co., in press.

Schroer, J.A.: Mapping of antigenic determinants on the insulin molecule using hybridoma antibodies. In Hammerling, G.J., Hammerling, U., and Kearney, J.F. (Eds.): Monoclonal Antibodies and T Cell Hybridomas. New York, Elsevier North Holland, in press.

LABORATORY OF IMMUNOLOGY
1981 Annual Report
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PHS-NIH
Summary Statement
Office of the Chief
Laboratory of Immunology
October 1, 1980 through September 30, 1981

Introduction

The Laboratory of Immunology is concerned with the elucidation of the fundamental mechanisms underlying immunologic responses. In the last year, it has made rapid progress through the use of three new technologies which are creating a revolution in immunologic sciences. These are the use of monoclonal antibodies, the adaptation of techniques of molecular genetics to immunologic problems, and the use of long term lines of cloned normal and transformed lymphocytes. The continued use and the improvement of these approaches should allow solution of many of the major problems which have concerned immunologists and should provide important approaches to the more precise regulation of the normal and the disordered immune response.

Probes for Genes Uniquely Expressed in B or T Lymphocytes

Subsets of lymphocytes differ from one another because of differences in the genes which are expressed in their mRNA and in their protein products. cDNA probes specific for these uniquely expressed genes have been developed by Laboratory of Immunology scientists. The approach used involved the preparation of mRNA from B and T cell tumors. The B cell mRNA was then used to generate a ^{32}P-labelled cDNA. This cDNA was then hybridized with a large excess of T cell mRNA. By removing the double stranded material, those cDNA molecules which recognize mRNAs expressed in both B and T cells are depleted. A repetition of this process followed by hybridization to B cell mRNA and selection of double stranded material produces cDNA which hybridizes almost completely with mRNA from B cell tumor lines or from normal B cells but to a limited degree, or not at all, with mRNA from T cell tumor lines or thymocytes. Based on the kinetics of hybridization of selected and unselected probes, it appears that the B and T cell tumor lines differ from one another in the expression of one to two percent of their genes. This would suggest that they may differ in the expression of one hundred to two hundred genes. A series of clones produced from total B cell cDNA have been selected with the "specific" B cell cDNA. Many of these clones hybridize with mRNA from B cell but not T cell tumors. A similar approach to identify T cell specific genes has been made. This approach should allow a definition of the genetic basis of phenotypic differences between closely related immunocompetent cells and may allow the isolation of genes which are normally expressed in mRNA at low copy numbers. (Davis, Cohen, and Paul, LI, NIAID; Kim, LMI, NIAID.)

The bas Mutation Limits the Numbers of Kappa-Positive B Cells but Not Pre-B Cells

Rabbits which are homozygous for the bas mutation produce serum immunoglobulins which are almost exclusively of the lambda light chain type. These rabbits make a small amount of kappa light chain which is b9-like and an antisera specific for the b9-like kappa chain of *bas* rabbits has recently been developed. Rabbits which are of the b^4/b^{bas} genotype express very small numbers of B lymphocytes bearing b9-like kappa chains. By contrast, examination of pre-B cells, which lack membrane immunoglobulin but possess cytoplasmic immunoglobulin, indicates that the number of pre-B cells expressing the b9-like kappa chain characteristic of *bas* were comparable to the number of b9-positive pre-B cells in a normal b^4/b^9 heterozygotes. Thus, the *bas* mutation controls either the transition of pre-B cells into B cells or the subsequent stimulant driven expansion of these cells. It has also been shown that very few of the immature B cells of adult bone marrow in b^4/b^{bas} heterozygotes express b9-like kappa chains suggesting the defect is expressed at the time of or very soon after the development of pre-B cells into B cells. The continued analysis of the defect in the bas mutant should allow a more precise understanding of the developmental processes in the B cell lineage. (Mage, LI, NIAID; Gathings and Cooper, Univ. of Alabama.)

The Role of Phospholipid Methylation in Lymphocyte Activation

Although many agents are known which can activate individual immunocompetent cells, relatively little is known about the chemical events which lead to activation. Recent demonstration of the importance of phospholipid methylation in many membrane triggered events has suggested that such mechanisms might also be important in receptor-based activation of lymphocytes. During the last two years, it has been shown that agents, such as lectins or anti-immunoglobulin antibodies, which bind to lymphocyte membrane receptors result in the prompt methylation of phosphatidylethanolamine to form lecithin. This methylated phospholipid is then degraded to arachidonic acid and lysolecithin, which appears to be a critical step in the subsequent activation of the cells. During the past year, it has been shown that methylation inhibitors block both the methylation of phosphatidylethanolamine and the activation of lymphocytes with similar dose-response curves. Similarly, the increase in calcium flux which has been previously shown to follow lectin binding appears to be important for the activation of phosphalipase A_2, a Ca-dependent enzyme. Phospholipase A_2 appears to be involved in the breakdown of lecithin. Recently, it has been shown that phospholipase A_2 is bound to a 40,000 dalton glycoprotein inhibitor, which may be identical to lipomodulin. Indeed, antibodies to lipomodulin block the proliferative response of lymphocytes to concanavalin A. It thus seems likely that an important mechanism in the activation of lymphocytes involves the activation of the arachidonic acid metabolism pathways by methylated phospholipids. Lipomodulin may prove to be a critical regulatory element controlling these important events. A fuller understanding of the detailed metabolic steps which underly lymphocyte activation should make precise control of this process a realistic possibility. (Iwata and Waxdal, LI, NIAID; Hirata and Axelrod LCM, NIMH.)

Long Term Lines of Non-transformed Human and Mouse B Lymphocytes

The direct study of the activation and the induction of tolerance in individual B lymphocytes has been hampered because normal B lymphocytes are a highly heterogeneous collection of cells of distinct specificities and distinct states of activation. Long term lines of homogeneous, immunocompetent B lymphocytes would provide a powerful tool for the study of these problems. During the past year, Laboratory of Immunology scientists have developed techniques to grow human and mouse B lymphocytes in culture for periods of greater than ten months and have succeeded in establishing cloned lines of human B lymphocytes. Long term human B lymphocyte lines have been established by culturing T cell-depleted populations of peripheral blood mononuclear cells with mitogen in suspension culture and then in soft agar. Colonies which form in the soft agar are picked and grown in suspension culture with a growth factor and irradiated cells. After these cells pass through a phase of "crisis," they can be maintained by feeding with growth factor alone. Such cells are membrane Ig bearing, lack the Epstein-Barr nuclear antigen, have 46 chromosomes, and can be stimulated to secrete immunoglobulin by certain growth factor preparations. Lines have been prepared from limiting dilution clonings which appear to represent cloned B lymphocyte populations.

Mouse B lymphocytes have also been grown in long term culture. Purified mouse splenic B lymphocyte populations were grown in lipopolysaccharide containing cultures for one to four weeks. The cells were then transferred into a medium containing a growth factor obtained from the T cell line, EL-4. The cells can be maintained by periodic feeding with this factor for periods of up to ten months; although their growth rate is still quite slow, sufficient numbers of cells can be obtained for certain experiments. The cultured cells express mIgM and Ia; they lack mIgD, mIgG, and Thy 1. When stimulated with a growth factor containing "T cell replacing factor (TRF)," the cells synthesize between 50 and 500 ng Ig 10^5 cells/3 days. Efforts to identify the growth factors important in propagating these cells are now in progress. Preliminary results indicate the existence of a "B cell growth factor" (BCGF) which is physically distinguishable from interleukin-2. Efforts to purify and characterize this molecule are now in progress. (Sredni, Howard, and Paul, LI, NIAID; Sieckmann, Naval Medical Research Institute; Farrar, National Institute for Dental Research.)

Individual Ia Molecules Display Several Distinct Restriction Specificities Controlling Responses to Specific Antigens

The capacity of an important set of T lymphocytes to respond to antigen requires them to recognize both antigen and an Ia molecule on an antigen-presenting cell. Furthermore, Ir gene control of the immune response stems from a non-permissiveness in the formation or recognition of certain antigen-Ia molecule pairs. One of the most powerful lines of evidence leading to these conclusions has been provided by the capacity of anti-Ia antisera to block antigen-specific T cell responses. The analytical power of this approach has recently been markedly improved by the use of monoclonal antibodies directed at individual determinants on Ia molecules and by the use of long term lines of antigen-specific T cells directed at individual determinants. Using such systems, Laboratory of Immunology scientists have now shown that a monoclonal anti-Ia antibody directed to a single determinant on a given Ia molecule will block some, but not all, responses in which that Ia molecule acts as a restriction element. Similarly, other monoclonal antibodies, specific for distinct determinants on the same Ia molecules, block responses of other T cell lines which recognize the same or other antigens in association with that Ia molecule. Thus, distinct sites on individual Ia molecules are involved in responses to different antigens or to different determinants on the same antigen. This suggests either that there are several distinct sites at which Ia molecules may interact with antigen or that there are several distinct self-receptors, capable of recognizing distinct determinants (or restriction specificites) on an Ia molecule. (Clark and Shevach, LI, NIAID.)

The Immunogenicity of Cytochrome C Depends upon Two Distinct Structural Elements

The immune response of T lymphocytes is regulated by specific immune response (Ir) genes encoded in the I region of the major histocompatibility complex (MHC). Such T lymphocytes recognize antigen in association with Ia antigens, which are products of I-region genes. Indeed, these two phenomena are almost certainly alternative aspects of the same fundamental process and an increasing body of evidence indicates that Ia antigens are the Ir gene products.

If Ia antigens exert their function in regulating immune responses by recognizing and reacting with antigen, then there should be specific sites on antigen which constitute the determinants recognized by Ia antigens. In addition, there must be sites on these antigens recognized by T cell receptors. Thus, theories of Ir gene control of immune response which posit an active antigen-binding step by Ia molecules require two recognition sites on antigen. Laboratory of Immunology scientists have obtained evidence for the existence of two such sites by studying immune responses of mice to evolutionary variants of cytochrome c and their amidinated derivatives. One site, controlling immunogenicity of the C terminal peptide of pigeon cytochrome c for B10.A mice, receives an important contribution from glutamine at residue 100 and this site seems to be subject to MHC genetic control since the response of strains of other MHC types is largely determined by this site. However, amidination of a critical lysine (or set of lysines) in this C terminal peptide creates a non-cross reactive antigen, which nonetheless shows precisely the same genetic control pattern. These results are consistent with the idea that the lys/amidinated lys site is critical to recognition by the T cell receptor and the glu-100 site is critical to Ia determined recognition. More detailed studies of the fine specificity of these and related systems will be important in determining whether this explanation for Ir gene function can be generalized. (Hansburg, Schwartz, and Inman, LI, NIAID; Appella, LCB, NCI; and Hannum and Margoliash, Northwestern University.)

Cloned T Lymphocyte Lines Specific for Antigen and Self-Ia Also Recognize Alloantigen

One of the most provocative findings of cellular immunogenetics is the appreciation of the very high frequency of T cells specific for MHC encoded alloantigens. Indeed, frequencies as high as 5% of T cells specific for antigens of any individual foreign haplotype have been reported. During the past year, Laboratory of Immunology scientists have shown that one important explanation of this rests in a high degree of cross-reactive recognition of alloantigens by T cells specific for foreign antigens and self Ia molecules (restriction elements). Cloned lines of T lymphocytes specific for dinitrophenylated-ovalbumin (DNP-OVA) were prepared from B10.A mice. These cells show the now classical phenomenon of MHC restriction. That is, their activation depends upon the presentation of DNP-OVA to them on antigen-presenting cells expressing I-region molecules of B10.A mice. Cells from 38 independent clones were tested for alloreactivity against spleen cells of mice of 8 independent haplotypes. No DNP-OVA was present in these cultures. Eight clones (21%) demonstrated alloreactivity in this small sample. Four of the clones proliferated in response to B10.S spleen cells and two to B10.D2 cells suggesting that the allostimulation specificity is non-random. The simplest explanation of these results is that T cell receptors specific for "self + X" (i.e., B10.A restriction elements and DNP-OVA) cross react with alloantigens (e.g., B10.S Ia antigens). This has substantial significance for the understanding of the nature of T cell receptors and the evolutionary forces which have shaped their specificity. (Schwartz and Sredni, LI, NIAID.)

The Determinant Recognized in the Syngeneic Mixed Lymphocyte Response Is a Self-Ia Molecule

T lymphocytes from individual humans and animals proliferate when stimulated with certain Ia-positive cells obtained from the same individuals or from individuals syngeneic to the T cell donor. Since the magnitude of this autologous or syngeneic mixed lymphocyte response is depressed in certain autoimmune diseases, it has been proposed that this response may have an important regulatory role. Others, however, have pointed out that the syngeneic mixed lymphocyte response might represent a response against an antigen found in the tissue culture medium or expressed on the stimulatory cells (e.g., a virus) and of no special physiologic significance. Thus, it is of critical importance to identify the stimulatory antigen in the syngeneic mixed lymphocyte response. Laboratory of Immunology scientists have approached this problem by using highly defined tissue culture medium and sera from syngeneic donors as well as culture conditions which do not support *in vitro* primary responses to rule out the contribution of exogenous antigens to this response. In addition, mice which receive limited antigen exposure *in vivo* (germ free mice) or which are incapable of *in vivo* responses because they have Ia discordant developmental micro-environments and antigen-presenting cells (A→B irradiation chimeras) yield strong syngeneic mixed lymphocyte responses, indicating that the proliferation does not represent the response of previously primed T cells. Finally, through the use of F_1→ parent chimeras and *in vitro* selection experiments, self-Ia molecules are strongly implicated as the actual stimulatory antigen. (Glimcher, Schwartz, and Green LI, NIAID; Longo, Medicine Branch, NCI.)

Cells Mediating Syngeneic Mixed Lymphocyte Responses Are Also Capable of Specifically Responding to Antigen

T lymphocytes obtained from human, mouse, and guinea pig sources can proliferate when stimulated with Ia-bearing cells derived from syngeneic individuals, or, indeed, from the donor of the responsive T cells. As described in the previous portion of the Summary, the stimulatory antigen is almost certainly the self-Ia molecule itself. Laboratory of Immunology scientists have now obtained clear evidence that many or all of the cells participating in the syngeneic mixed lymphocyte response can respond to some antigen in association with self-Ia molecules. The experiments which demonstrated this involved the priming of (2 x 13)F_1 hybrid guinea pigs with individual antigens. T lymphocytes were prepared from these animals, thoroughly washed and then stimulated in syngeneic mixed lymphocyte responses using cells of one of the two parents. That is, T cells capable of reacting against Ia molecules of one of their parental types were positively selected. These T cells were then assessed for responsiveness to the antigen to which the T cell donor had been primed. When so tested, it could be shown that such selected T cells preferentially responded to antigen presented by antigen-presenting cells of Ia type of the stimulator cells used in the positive selection by syngeneic mixed lymphocyte stimulation. This strongly implies that the syngeneic mixed lymphocyte response represents a polyclonal stimulation of antigen-specific, MHC restricted T cells through their "partial specificity" for self-Ia molecules. (Dos Reis and Shevach, LI, NIAID.)

Regulatory Idiotopes and the Immune Network

Idiotypic determinants (idiotopes) expressed on antibody molecules and on receptors of lymphocytes may act as sites for the regulatory action of certain types of lymphocytes and antibodies. Jerne has suggested a theory in which the elements of the immune system are interconnected by interactions specific for idiotopes, forming an "immune network." Scientists in the Laboratory of Immunology have examined certain aspects of such immune networks by immunizing BALB/c mice with a homogeneous antibody (Ab_1) in the form of a BALB/c myeloma protein (A48), expressing a well defined idiotype and a binding site for bacterial levan. The antibody produced in response to immunization with A48 is specific for the A48 idiotype and is termed Ab_2. In turn, Ab_2 can be used to immunize BALB/c mice to produce anti-Ab_2 (Ab_3) and Ab_3 can be used to induce anti-Ab_3 (Ab_4). Since A48 is a BALB/c myeloma protein and each of these immunizations is done in a BALB/c mouse, this artificial production of network elements should reveal features of their specificity. The most striking finding to emerge from this work is that Ab_4 binds Ab_1. That is, that Ab_3 and Ab_1 share a similar idiotype (or at least one similar idiotope). On the other hand, Ab_3 has little if any binding activity for bacterial levan, indicating that most of the molecules bearing this "Ab_1-like idiotope" fail to bind levan. This result has led to the proposal of a theory ("The Regulatory Idiotope Theory") in which the network is viewed as largely reflective in nature. That is, Ab_1 expresses a binding site (p_1) and an idiotope (i_1). In response to Ab_1, a family of Ab_2 molecules are produced which possess a binding site specific for i_1, (p_2) but do not possess any common idiotope. Thus, when Ab_2 appears naturally or is artificially administered, it activates i_1 rather than p_3 bearing cells. However, i_1 is viewed as being widely expressed and found on antibodies of many distinct specificities. Thus, the immune system is subject to a special kind of receptor based regulation in which lymphocytes bearing receptors which express widely shared idiotopes (regulatory idiotopes) are regulated and interconnected by anti-idiotope specific elements but in which the arborization of the regulatory system is very limited. (Paul and Heber-Katz, LI, NIAID; Bona, Mount Sinai Medical School.)

Interleukin 1, or a Closely Related Molecule, Stimulates Fibroblast Proliferation

Fibrosis is an important feature of many immunopathological conditions. A factor in mixed lymphocyte culture supernatants has been previously shown to stimulate fibroblast proliferation and collagen biosynthesis. Laboratory of Immunology scientists have undertaken a more careful analysis of this soluble factor and have shown that the active material is a 15,000 dalton molecule which can complex with albumin dimers. On isoelectric focusing, this material has pI's of 7.0, 6.2, and 5.4. Each fraction contains interleuken 1 (IL-1) activity as assessed both by a thymocyte proliferation assay and by the capacity to induce interleukin-2 (IL-2) production by a cloned T lymphoma line. Moreover, partially purified human and mouse IL-1 stimulates fibroblast proliferation, whereas IL-2 has no activity. These results strongly implicate IL-1 as a product of immune responses which is important in the fibrosis seen in chronic inflammatory states. (Schmidt and Green, LI, NIAID; Mizel, Pennsylvania State University.)

Administrative, Organization and Other Changes

The Laboratory of Immunology continues to play an important role in the training of young scientists. During the past year Drs. Daniel Hansburg, Robert Clark, and Louis Matis completed research associateships. Dr. Toby Hecht completed an appointment as a Senior Staff Fellow and Drs. Shunichi Kumagai, Hamida Abdi, Josianne Wantyghem and Joe Chiba completed training as Visiting Fellows. In addition, Dr. Harley Tse and Dr. Shuresh Shukla completed periods as Guest Workers and Dr. Kenji Adachi completed his appointement under the Intergovernmental Personnel Act (IPA). Each of these young scientists made very substantial contributions during their working period in the Laboratory of Immunology.

Dr. Jonathan Ashwell and Dr. John Ansel joined the Laboratory as Medical Staff Fellows and Dr. Hyun Lillehoj was appointed as a Staff Fellow. Drs. George Dos Reis, I-Yu Sillers, Claudine Kieda, Susumu Nishinarita, and Kenji Nakanishi were appointed as Visiting Fellows; and Drs. Mark Davis and Nancy Francis entered the Laboratory as Guest Workers.

Mrs. Margaret Anglin resigned as Laboratory Chief's Secretary and Mrs. Mary K. McMahon was appointed to this position.

Honors, Awards and Scientific Recognition

Professor Baruj Benacerraf, of Harvard Medical School and former Chief of the Laboratory of Immunology, was awarded the Nobel Prize for Physiology or Medicine during the past year. An important part of the research which resulted in this honor was carried out by Dr. Benacerraf with colleagues in the Laboratory of Immunology.

Current members of the Laboratory of Immunology play important roles in the national and international scientific community. Included in this is service on editorial boards of important journals. Dr. Paul is a member of the editorial board of the Journal of Immunology; Drs. Shevach and Schwartz are associate editors of this journal. Drs. Shevach and Mage are members of the editorial board of the Journal of Immunologic Methods. Dr. Shevach is also a referee editor of the Proceedings of the Society for Experimental Biology and Medicine and a member of the editorial board of Cellular Immunology. Dr. Mage is the Chairman of the editorial board of Federation Proceedings. Dr. Schwartz is a member of the editorial board of Stem Cells. Dr. Inman is an advisory editor of Molecular Immunology and Dr. Green is a member of the editorial boards of Immunologic Communications and of Clinical Immunology and Immunopathology. Dr. Paul is an advisory editor of the Journal of Experimental Medicine, a member of the editorial boards of the Scandinavian Journal of Immunology, Immunological Reviews, CRC Critical Reviews in Immunology, Human Immunology, and the Journal of Clinical Immunology. He is an associate editor of Immunological Communications.

Ms. Rose Lieberman was awarded the Distinguished Service Award of the Department of Health and Human Services, and the NIH Director's Award. She chaired a Topical Review Session at the ICN-UCLA Conference on Immunoglobulin Idiotypes and their Expression in Salt Lake City, Utah.

Dr. Paul served as President of the American Society for Clinical Investigation. He was elected to the Council of the American Association of Immunologists and to the Board of Directors of the Foundation for Advanced Education in the Sciences. He received the Distinguished Alumnus Medallion for 1981 from the Alumni Association of the State University of New York, Downstate Medical Center. He was named U.S. Chairman of the Immunology Board of the U.S.-Japan Collaborative Medical Science Program and was Co-Chairman of the NIAID Study Group on Immunology. He is a member of the Scholars Selection Committee of the Fogarty International Center, of the Committee on Promotions and Tenure, NIAID, of the Scientific Review Board of the Howard Hughes Medical Institute, of the Scientific Board of Visitors of the Oklahoma Medical Research Foundation, and of the Committee on Personnel for Research of the American Cancer Society. During the past year, he completed terms as Co-Chairman of the Program Committee of the American Association of Immunologists and as a member of the Fellowships Sub-Committee of the Arthritis Foundation.

Dr. Paul presented a summary lecture at the ICN-UCLA Conference on Immunoglobulin Idiotypes and their Expression in Salt Lake City, was a session chairman at the Gordon Conference on Immunochemistry and Immunobiology, an invited mini-symposium speaker at the FASEB meeting, an invited speaker at the U.S.-Japan

Immunology symposium in Tokyo, and an invited speaker at the Workshop on Lymphocyte Cloning, organized by the Given Institute in Aspen, Colorado.

Dr. Green received the Philip Hench Award of the Association of Military Surgeons of the United States, completed a term as Chairman of the Program Committee of the American Association of Immunologists and was named to the executive board of the American Academy of Civil Service Physicians. He completed a term as a member of the Transplantation Immunology Committee of NIAID. He presented an invited address at the International Systemic Lupus Erythematosus Symposium in Philadelphia and chaired a workshop at the Leukemia Markers Conference in Vienna, Austria.

Dr. Mage was elected President of the District of Columbia Chapter of Sigma Xi and was nominated as Section Representative in Immunology of the American Society for Microbiology. She was the Introductory Speaker and Co-Convenor of the American Society for Microbiology symposium, "Cell Surface Receptors Involved in the Immune System," and was a chairman of a topical review session at the ICN-UCLA meeting on Immunoglobulin Idiotypes and their Expression in Salt Lake City, Utah.

Dr. Shevach presented major addresses at the Leukemia Markers Conference in Vienna, Austria and the meeting on Fundamental Mechanisms in Human Cancer Immunology, in Galveston, Texas. He chaired the minisymposium on Macrophages as Antigen-Presenting Cells at the FASEB meeting, and was chairman of a session at the Workshop on Mucosal Immunity, in Bethesda, Maryland.

Dr. Schwartz presented invited lectures at the New England Conference of Immunologists, in Woods Hole, Massachusetts, at the Mid-Winter Conference of Immunologists at Asilomar, California, at the annual meeting of the American Rheumatology Association, at the Gordon Conference on Immunochemistry and Immunobiology, and at the Workshop on Lymphocyte Cloning at the Given Institute, Aspen, Colorado.

Dr. Inman was an invited speaker at the Fourth International Symposium on Affinity Chromatography and Related Techniques at Veldhoven, The Netherlands.

Dr. Waxdal received a fellowship from the Japanese Society for the Promotion of Science to visit and lecture at several Japanese universities.

In addition, laboratory members presented seminars and lectures at universities and research institutes, both in the United States and abroad.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00030-13 LI

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)
Antigen Recognition and Activation of Immunocompetent Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: William E. Paul, M.D., Chief, LI/NIAID

Other Investigators:

T. Hecht, Ph.D., Sr. Staff Fellow; P. Mongini, Ph.D., Guest Worker; E. Heber-Katz, Ph.D., Guest Worker; J. Kung, Ph.D., Visit. Fellow; M. Howard, Ph.D., Guest Worker; A. DeFranco, Ph.D., Guest Worker; B. Sredni, Ph.D., Visit. Associate; M. Davis, Ph.D., Guest Worker; D. Cohen, M.D., Res. Associate; L. Glimcher, M.D., Res. Associate; R. Lieberman, M.A., Sr. Investigator, LI/NIAID. C. Bona, M.D., Ph.D., Dept. Microbiol., Mt. Sinai Med. Ctr., New York; D. Sieckmann, Ph.D., Div. of Immunol., NMRI, Bethesda, Md.; J. Mond, M.D., Ph.D., Dept. of Med., USUHS, Bethesda, Md.; J. Farrar, Ph.D., NIDR; A. Ahmed, Ph.D., Merck Sharp & Dohme Res. Lab, Rahway, N.J.

COOPERATING UNITS (if any) Dept. of Micro., Mt. Sinai Med. Ctr, N.Y.; Naval Med. Res. Institute, Bethesda, MD; Uniformed Services University of the Health Sciences, Bethesda, MD; Merck, Sharp and Dohme Res. Lab., Rahway, N.J.

LAB/BRANCH
Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectous Diseaes, National Institutes of Health, Bethesda, MD 20205

TOTAL MANYEARS: 7	PROFESSIONAL: 4½	OTHER: 2½

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

B lymphocyte functional heterogeneity and activation requirements have been studied by a variety of coordinated approaches. New markers characterizing various mouse B cell subsets have been identified by the use of monoclonal antibodies and the functional properties of cells bearing these markers are being elucidated. Early events in B cell activation are being studied by the use of highly purified populations of defined B lymphocytes grown in both serum free and conventional medium. Techniques to grow long term lines of non-transformed human and mouse B lymphocytes, and for the cloning of human B cells have been developed. A molecular genetic approach to the characterization of genes which determine the differentiated state of given lymphocyte classes is now being developed. These approaches used together with the resources provided by mice with a B cell defect determined by the xid gene have provided an overall strategy to elucidate key elements in the biology of B lymphocytes.

Objectives

The thymus-independent (B) lymphocytes are one of the two major subsets of lymphocytes; their principal function is the production of antibodies. Although the chemistry and molecular genetics of their products, immunoglobulins, are known in considerable detail, our understanding of their functional heterogeneity and their activation requirements is still quite limited. Such information would be of great importance in development of effective ways to control antibody responses to a variety of pathogenic agents.

The Laboratory of Immunology has developed an extensive program aimed at delineating some of the key features regulating B lymphocyte development and activation. Particular emphasis has been placed on development of markers which characterize various subsets of B lymphocytes, on the activation requirements and of cell cycle analysis of purified B lymphocytes stimulated with anti-immunoglobulin antibodies and with bacterial lipopolysaccharide, on the development of techniques to propagate non-transformed B lymphocytes in long term culture and to clone these cells, and on molecular genetic approaches to characterize the genes responsible for the unique differentiated states of distinct B and T cell subsets.

Methods Employed

Virtually all standard techniques of cellular immunology and immunogenetics have been employed in these experiments. Techniques of special interest include production of monoclonal antibodies, analysis of membrane-antigen expression using a fluorescence activated cell sorter (FACS-II Becton-Dickinson, Mountain View, CA), soft agar colony formation, long term culture and cloning of B lymphocytes, and molecular genetic techniques to analyze expressed genes in B and T lymphocytes.

Results

A. Monoclonal Antibodies to Distinct Mouse B Lymphocyte Antigens

A series of (rat x mouse) somatic cell hybrids secreting rat antibodies specific for mouse B cell antigens has been produced. Among these, several are of special interest. One, designated Bet-1, is a rat anti-mouse IgM antibody which identifies an IgM allotypic determinant, Igh-6.5. This is the first allospecific monoclonal anti-IgM antibody which has been developed and has value both for characterization of *Igh* gene haplotypes of mice and as a marker in cell transfer experiments. Using Bet 1, we have identified four allelic forms of the *Igh6(u)* gene, *a*, *b*, *e*, and *n*. IgM of mice of the *a* type is strongly reactive with Bet 1, that of *b* and *n* mice is weakly reactive, and that of *e* mice, unreactive.

A second important antibody is 14G8. It recognizes an antigenic determinant expressed on approximately 30% of B lymphocytes from normal mice and on more than 60% of B lymphocytes from mice with the *xid* determined immune defect. Cells which express this antigen are found in high frequency in spleens of neonatal mice and resemble cells from mice with the xid determined defect in the relative amounts of surface IgM and IgD they express. Functionally, B cells which express the antigen recognized by 14G8 proliferate in response to both anti-IgM (anti-μ) antibodies and to LPS. By contrast, cells lacking this antigen respond well to anti-μ) and poorly, or not at all, to LPS. These results are somewhat paradoxical in view of our initial conclusion that 14G8 identified $Lyb5^-$ B cells since previous work has shown that treatment with anti-Lyb5 and complement completely eliminated proliferative response to anti-μ. Techniques to purify cells on 14G8 plates have been developed. Studies are now in progress aimed at determining the relationship between cells positive for the antigen recognized by 14G8 and cells expressing Lyb5.

Other important monoclonal reagents are 14D10, which recognizes an antigen expressed on all B lymphocytes and on a subset of $Lyt2^+$ T cells; anti-ThB, a reagent which reacts with most thymocytes and most B cells but not peripheral T cells; and 14C8, which reacts with a very small number of spleen cells.

B. Activation of Resting B Lymphocytes

Populations of small, dense B lymphocytes have been prepared by eliminating T cells with anti-Thy 1 and complement and then selecting the appropriate B cells by density gradient separation using Percoll. If these cells are cultured in a modified Iscove's medium containing no serum, they retain their characteristic size for at least 48 hours. If anti-μ is added to the culture, all the cells enlarge and this enlargement begins almost immediately. Cells incubated with anti-μ for one hour are measurably larger than control cells. Enlargement, appears to represent the G_1 phase of the cell cycle;;it continues for approximately 30 hours. For at least 24 hours of this period, anti-μ is required and must be present continuously. If anti-μ is removed at any point during this period, the cells "freeze" at the size they were when the stimulant was withdrawn but will promptly recommence enlargement when anti-μ is re-added.

Although virtually all the B cells enlarge when stimulated with anti-μ, only 50 to 60% of them synthesize DNA and proliferate. That is, anti-μ can cause all cells to enter G_1 but only a portion enter S, under conventional stimulation. Preliminary experiments suggest that cells which normally do not enter S in response to anti-μ can be stimulated to do so by LPS.

Recently, we have employed a very rigorous protocol to prepare functionally pure B cells. Mice are treated with anti-thymocyte serum. Two days later, their spleen cells are removed and treated with monoclonal anti-Thy 1, anti-Lyt 1, and an antibody capable of lysing most of null cells. The remaining cells are then passed over Sephadex G-10 which depletes macrophages and activated B lymphocytes. Such cells, if cultured at 10^5 per well, proliferate little or not at all when stimulated with either anti-μ or LPS. If interleukin-1 (IL-1) is added to these cultures, LPS is capable of stimulating a response. If a growth factor containing supernatant prepared from a T cell tumor line, EL-4, is added, proliferation also occurs. Addition of both factors creates a summation in the amount of thymidine uptake, suggesting that the cells treated with LPS that are stimulated to divide by IL-1 are distinct from those stimulated to divide by EL-4 supernatant.

Futhermore, DNA synthesis by cells stimulated by anti-μ can be induced with EL-4 supernatant but not by IL-1. Since anti-μ responsiveness is a unique property of Lyb5$^+$ B cells, this suggests that EL-4 supernatant acts principally on Lyb5$^+$ B cells stimulated with either LPS or anti-μ. IL-1, by contrast, acts mainly on Lyb5$^-$ B cells. This is supported to some extent by the finding that B cells from mice with the xid determined immune defect, which are exclusively Lyb5$^-$, can respond to LPS plus IL-1, but not to LPS plus the EL-4 supernatant.

Collaborative efforts with Dr. John Farrar of NIDR have been initiated to characterize the B cell growth factor in EL-4 supernatant. Initial results indicate that this activity can be separated from interleukin-2 (IL-2) activity by SDS-polyacrylamide gel electrophoresis and that the B cell growth factor has a lower molecular weight than IL-2.

C. Long Term Lines of Non-Transformed B Lymphocytes

Propagation of non-transformed human and mouse B lymphocytes over extended periods (> 10 months) has now been achieved although by two rather different strategies. Mouse cells to be used for producing long term lines are prepared by treatment with anti-Thy 1 and complement, followed by positive selection of Ig-bearing cells on the FACS. Recently, we have also employed the method outlined previously for preparation of functionally pure B cell populations. These cells are cultured with LPS for a period of one to four weeks and are then transferred into a culture medium containing a source of B cell growth factor. They can be maintained for extended periods of time by feeding every three days with growth factor and splitting as necessary. With current growth factor preparations, the doubling time of these cells is quite long. The cells are membrane IgM$^+$, IgD$^-$, IgG$^-$; they express Ia antigens but lack Thy 1. They continue to grow if transferred from a source of B cell growth factor into an IL-1 containing medium. In either situation, they secrete little or no immunoglobulin (< 10 ng/ml per 10^5 cells in 3 days). However, if transferred to a complex supernatant containing T cell replacing factor (TRF), in addition to other factors, they secrete amounts of Ig ranging from 50 to 500 ng/ml per 10^5 cells in three days. These cells are unresponsive to LPS.

Long term lines of human B lymphocytes have been prepared by partially purifying B cells from human peripheral blood, growing them for three days in suspension culture in the presence of phytohemagglutinin (PHA), protein A, or pokeweed mitogen (PWM), and then transferring them to the upper layer of a two layer soft agar system. The mitogen used in the initial suspension culture is incorporated in the lower layer. Colonies form 3 to 7 days later. They can be picked, placed in suspension culture, and expanded using a growth factor-containing supernatant prepared by stimulating human peripheral blood mononuclear cells with PHA or PWM for 72 hours. The cells generally enter a state of "crisis" when transferred to larger culture wells, which can be overcome in some cases by adding irradiated human mononuclear cells. Once this crisis is past, the cells can be propagated with growth factor containing supernatants only.

The cells express mIg, lack the Epstein-Barr nuclear antigen, and have 46 chromosomes. In some growth factor preparations, they secrete large amounts of Ig, while in others they make little or no Ig. The cells can be cloned by limiting dilution. In such situations, the frequency of wells in which cell growth occurs conforms to a Poisson distribution with a single unit required for growth. The cloning efficiency is ∿1 in 3. B cell lines of this type have been maintained for over 13 months and have been successfully frozen and recovered.

D. Analysis of Genes Uniquely Expressed in B or T Lymphocytes

Subsets of lymphocytes differ from one another because of differences in the genes which are expressed in their mRNA and in protein products. An approach to determining the number and the character of the genes determining the unique properties of B and T lymphocytes has been initiated. mRNA has been isolated from a B cell tumor line, BAL-17, and from a T cell line, EL-4. The B cell mRNA has been used to generate a ^{32}P-labelled cDNA. This cDNA is then hybridized with a large excess of mRNA from the T cell tumor. By removing double stranded material on a hydroxyapatite column, those cDNA molecules which recognize mRNAs shared by B and T cells are depleted. This process is repeated and the resulting single stranded cDNA is then hybridized to B cell mRNA to positively select "B cell specific" cDNA. Preparations of this type hybridize almost completely with mRNA from BAL-17 and to an extent of approximately 5% with mRNA from EL-4. The cDNA hybridizes almost completely with spleen cell mRNA suggesting it contains few if any tumor specific cDNAs. It also hybridizes with spleen cells from nu/nu mice, but reacts with thymocyte mRNA very poorly. mRNA from other B cell lymphomas can bind ∿80% of the cDNA but mRNA from pre-B cell (Abelson) lines and from myeloma lines reacts only partially. An analysis of the kinetics of binding of the "B cell cDNA" suggests that it recognizes 50 to 100 distinct mRNA families (i.e. genes) expressed in B cells and not T cells. A series of clones has been prepared from this "B cell cDNA" and an analysis of these distinctive genes, as well as genes distinguishing distinct B cell differentiated states (i.e., pre-B cells and mature B cells) is now in progress.

A similar strategy has been used to prepare a "T cell specific cDNA." We estimate that it, too, identifies ∿ 100 "T cell-specific" genes.

E. Antigen-Presentation by B Cell Tumor Lines

A reliable antigen-presenting tumor line would be of great value for analyzing the process through which antigen-presentation to histocompatibility-restricted, antigen-specific T cells proceeds. We have previously observed that a cell tumor line, A-20, had some antigen-presenting capacity but that the magnitude of the responses it stimulated was limited and its capacity to stimulate depended upon the presence of live, non-irradiated spleen cells in the culture. We have recently reexamined this issue but have used A-20 to stimulate T cells in the presence of IL-1. We find that primed cells which have been rigorously depleted of antigen-presenting cells can be stimulated to proliferate by antigen and A-20, if IL-1 is present. Indeed, in the presence of IL-1, A-20 is a substantially better stimulant than irradiated spleen cells. A-20 also stimulates mixed lymphocyte responses in the presence of IL-1. Analysis of this effect indicates that A-20 shows histocompatibility-restricted antigen-presentation and that a cloned line of T cells with mixed lymphocyte responsiveness can be stimulated with A20. We have found that some, but not all, other B cell lines that are Ia^+ stimulate responses in presence of IL-1. A-20 and other lines with presentation function should be valuable for studies of the molecular basis of antigen-presentation.

F. The "Regulatory Idiotope" Concept

We have recently examined idiotype-anti-idiotype systems in mice by immunizing syngeneic (BALB/c) mice with a levan-binding myeloma protein, A48, and preparing anti-idiotype directed against A48. We term A48 Ab_1 and the anti-idiotype Ab_2. Immunization with Ab_2 leads to the appearance of antibodies that bind Ab_2, but fail to bind levan. Thus, these molecules appear to be directed against the Ab_2 idiotype and could be designated Ab_3. However, antibodies to Ab_3 resemble Ab_2 in that they bind Ab_1 (i.e., A48). Thus, we suggest that Ab_3 really consists of a collection of molecules which share idiotypic determinants with Ab_1, although most do not bind levan. Indeed, we have proposed a version of the network theory in which only a limited set of idiotypic determinants, termed regulatory idiotopes, induce anti-idiotope responses. Such anti-idiotope antibodies generally do not, themselves, express regulatory idiotopes. Thus, such Ab_2 molecules mainly activate precursors expressing the Ab_1 regulatory idiotope. This suggests that the "network" is mainly reflective in character and that it may be organized into a series of relatively independent mini-networks.

Future Course of the Project

Continued emphasis will be placed on studies of the cell biology and molecular genetics of B lymphocytes. We will be especially interested in the inter-relationship of stimulants and growth factors in the activation of resting B lymphocytes in order to improve our techniques for long term growth and cloning of B cells, with emphasis on creating antigen-specific B cell lines. We will attempt to characterize a substantial number of the genes which distinguish B and T cells. Efforts to test some of the predictions of the regulatory idiotope concept are also planned.

Significance to Biomedical Research

These studies, aimed as they are at uncovering the fundamental cellular and genetic mechanism underlying antibody production, promise to provide new insights into the ability to more precisely control immune responses. In turn, this should allow one to amplify responses that are normally too limited to provide protection against certain classes of pathogenic microorganisms and tumors and to limit responses in instances of autoimmunity. Both of these goals clearly would be of importance in control of disease states.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00148-06 LI

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Lymphocyte Interactions, Receptors and Functions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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6	4	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to explore immunological mechanisms of disease and immunopathological processes. A failure of immune regulation is suspected to be involved in the super-normal production of auto antibodies in systemic lupus erythematosus (SLE). Treatment of normal T cells with serum from patients with SLE leads to increased proliferative and cytotoxic responses, indicating that such treatment has removed a naturally occurring suppressor cell. Absence of such suppressor cells in patients with SLE may lead to increased auto antibody formation. Immunologically mediated inflammation is often accompanied by the late onset of fibrosis. We have shown that supernatants from mixed lymphocyte reactions contain IL-1 or a closely related factor which is capable of inducing fibroblast proliferation. The local production of IL-1 by monocytes in areas of immunologically mediated inflammation may therefore be the reason for the fibrosis observed. Another pathological process that we are investigating is the mechanism through which P. falciparum infected erythrocytes attach to umbilical vein endothelial cells. Antibodies in the sera of infected monkeys block this interaction and may provide a means to explore the nature of the receptors on the infected RBC and the endothelial cells involved in this process.

Pathogenesis of Systemic Lupus Erythematosus (SLE)

Objective:

Patients with SLE have increased levels of auto antibodies which are thought to be responsible for many of the manifestations of this disease. The cause of this increased auto-antibody production is thought to rest in part in the dysregulation of certain cellular immune processes; more specifically an absence of regulatory suppressor T cells has been observed in this disease. One of the many auto antibodies present in SLE is directed against T cells and in this present investigation we wished to determine whether such antibodies could eliminate suppressor T cell functions from populations of normal lymphocytes.

Methods Employed:

Peripheral lymphocytes and plasma from patients with SLE, as well as those of normal subjects are used. For separation of T cells, Ficoll-Hypaque gradient techniques are used after rosetting with sheep red blood cells. Fc-receptor bearing T cells (T cells) are separated by rosetting with IgG antibody coated ox red blood cells. Trinitrophenyl sulfonic acid (TNPBS) is used to modify the surface of autologous cells.

After cultivation of T cells or non-T-cells with autologous TNP-modified cells, their proliferative and cytotoxic activities are measured by ^{3}H-thymidine uptake and ^{51}Cr release from TNP-modified autologous cells, respectively. The ability of lymphocytes from patients with SLE to perform these functions were compared to lymphocytes from normal individuals. In addition, plasma from patients with SLE was used together with complement to treat normal lymphocytes.

Major Findings:

Pure T cells obtained from the peripheral blood were examined for their proliferative and cytotoxic responses against trinitrophenyl (TNP)-modified autologous non-T cells. Primary and secondary proliferative T-cell responses to TNP-non-T cells were markedly impaired in patients with active systemic lupus erythematosus (SLE), as compared to normal subjects or patients with inactive SLE. In contrast, patients with active SLE had significantly greater cytotoxic activity against TNP modified autologous non-T cells. Thus, there is a dissociation between the proliferative and cytotoxic abilities of SLE T cells.

Patients with active SLE had impaired proliferative responses to non-modified autologous non-T cells (Auto-MLR). A significant negative correlation was observed between the degree of the auto-MLR and the degree of cytotoxic ability against TNP-modified autologous cells in patients with SLE. This observation suggests that cells capable of proliferating in the auto-MLR might regulate the generation of cytotoxic T cell responses against modified self.

The treatment of normal T cells with active SLE plasma led to the generation of secondary cytotoxic activities against TNP-modified autologous cells. These enhancing effects of active phase plasma on these proliferative and cytotoxic TNP responses were also seen when the T cells from inactive SLE patients were pretreated with their own active phase plasma. The SLE plasma-induced augmentation of proliferative TNP responses of normal T cells were inhibited by adding normal T cells. The above results suggested that treatment of normal T cells with active SLE plasma-killed subpopulations of suppressor T cells leading to increased TNP responses.

In a separate study we investigated whether the cloning frequency of B cells from peripheral blood was increased in patients with SLE. An increased ability to clone B cells from spleens of NZB/W mice had been previously reported. We have succeeded in generating B-cell colonies from freshly separated human peripheral blood using the double-agar layer technique and in expanding them in liquid culture for several weeks. Populations of cells enriched in B cells were placed into the upper layer with or without bacterial lipopolysaccharide (LPS). Although B-cell colonies were observed without LPS, addition of LPS to the cells in the upper agar layer increased the number of colonies. Also necessary for colony formation in the upper layer was the presence in the lower agar layer of helper factors. Two sources of these factors were used. The culture supernatant of phytohemagglutinin stimulated mononuclear cells or a mixture of purified T cells and phytohemagglutinin placed directly in the lower agar layer.

Using these techniques, peripheral blood lymphocytes from normal individuals and patients with active systemic lupus erythematosus (SLE) were studied for the generation of B-cell colonies. Significantly more B-cell colonies were observed in patients with SLE, using either kind of "helper" factor. T cells from normal individuals and patients were tested for their ability to help B-cell colony formation. The SLE T cells were found to be defective in this function relative to normal T cells. Thus, although B cells from patients with SLE were hyperactive in forming B-cell colonies, their T cells were defective in supporting the formation of B-cell colonies from normal individuals. The observation of a dual abnormality in patients with SLE (increased B-cell colony formation but decreased SLE T-cell support of normal B-cell colonies) suggests that SLE may be the result of an imbalance between increased B-cell function and decreased T-cell function.

These new techniques in which separate populations of immunologically competent cells are allowed to interact functionally under controlled conditions may provide a new way to study cellular relationships in normal immunological function as well as in disease states. It would therefore appear that patients with SLE have several types of unrelated immunological defects. The simultaneous presence of these several types of defects in a single individual may be necessary for the full expression of the disease.

Significance to Biomedical Research:

These observations suggest that the antibodies to T lymphocytes among the many auto antibodies found in patients with SLE, may play an important role in perpetuating the disease process by interfering with naturally occuring suppressor T cell functions, which interference may in turn lead to increased auto antibody production, thus perpetuating a disease producing cycle. The finding of an increased frequency of B cell colonies in patients with SLE strongly support the notion of a separate intrinsic B cell defect in this disease. Further study of the ability to form B cell colonies in patients with SLE who have varying degrees of disease activity may prove to be especially revealing. The removal of anti T cell antibodies which interfere with normally occurring suppressor function could be of benefit in this disease.

Project Description

The syngeneic mixed lymphocyte reaction (SMLR) is the in vitro proliferation that occurs when T lymphocytes are co-cultured with irradiated syngeneic non-T cells. This reaction has been shown to have immunological specificity and memory. In humans the SMLR has been shown to be absent in several diseases including the auto-immune disease lupus erythematosus (SLE). This is especially provocative since there is also evidence that the T lymphocyte that proliferates in the SMLR may act as a cell which suppresses the immune response. Because the SMLR may have important immunological significance a number of studies are being performed to further analyze this phenomenon.

Objectives:

A) Study of the nature of the target antigen being recognized on the stimulator cell by the autoreactive T cell.

Methods Employed:

T cells obtained from lymph nodes were used as the responding cells and BSA gradient separated syngeneic irradiated spleen cells were used as the stimulator cell population. The proliferation was measured by ^{3}H thymidine incorporation at 6 days.

Major Findings:

A system has been described which produces a murine syngeneic MLR comparable in magnitude to an allogeneic MLR. The responder cells in these cultures are T cells which exhibit the classic immunologic characteristics of both memory and specificity. Studies utilizing radiation-induced bone marrow chimeras of F_1-parent type indicated that similar to many other cell mediated immune responses, the response of the T lymphocytes in the SMLR was determined by the environment in which they matured. We took advantage of this observation to map the genes determining the self antigen which elicits T cell proliferation by utilizing responder T cells from F_1 radiation bone marrow chimeras and stimulator cells from H-2 recombinant strains. These studies revealed that the response of F_1 chimeras was dependent on antigens encoded in the K and/or I regions. In addition, blocking studies suggested that in the B10.A strain the target antigens were products of both the I-A and I-E subregions.

Objectives:

B) Examination of the function and specifity of the responding T cell in the SMLR.

Methods Employed:

Slope analysis and precursor frequency analysis of the responding T cell population indicates that 2 cells are involved in this reaction, each present in a frequency of $\underset{\sim}{\sim}$ 1/5000. Further analysis of these 2 cell populations will be undertaken by examining the SMLR in the thymus and spleen of radiation induced bone marrow chimeras. In addition, the autoreactive T cells will be cloned using techniques developed by B. Sredni. T cells are first stimulated in an SMLR by non T cells, isolated and cloned in soft agar and then grown up in liquid culture (with the help of T cell growth factors). These clones of T cells can then be tested for their ability to respond to syngeneic stimulating cells, allogeneic stimulating cells, as well as different protein antigens. In a related study, a line of SMLR T cells will be produced by fusing T cells from an SMLR culture with the T cell lymphoma BW5147. The large quantity of cells obtainable by such a method should enable us to produce antibodies against the receptor on the T cell that recognizes self Ia determinants.

Significance to Biomedical Research:

The syngeneic mixed lymphocyte reaction may be a manifestation of an important immunoregulatory mechanism. The selective loss of the SMLR but not the allogeneic MLR in patients with SLE, Hodgkins disease and primary biliary cirrhosis (in whom a loss of suppressor T cells is also observed) may be an important new clue as to the etiology of certain human autoimmune diseases. Equally, the existence of a T cell which appears to recognize self MHC determinants may allow us to isolate and identify the T cell receptor for self antigen.

Study of an in vitro model for the sequestration of Plasmodium falciparum malaria.

Project Description

It has been recognized since 1890 that the mature asexual forms of Plasmodium falciparum malaria, viz., trophozoites and schizonts, are absent from the peripheral circulation of infected hosts. Subsequent in vivo studies demonstrated that these forms adhere to deep venous endothelium and that attachment occurs at discrete points on the infected erythrocyte membrane known as knobs. Since this sequestration phenomenon may allow the parasite to elude destruction in the host reticuloenthelial system, in vitro studies designed to further investigate this phenomenon were undertaken.

Objective:

To establish an in vitro model for the sequestration of Plasmodium falciparum infected erythrocytes.

Methods Employed:

Plasmodium falciparum-infected erythrocytes were cultured according to the methods of Jenson and Traeger. Human umbilical vein endothelial cells were obtained according to the method of Jaffe, et al. A suspension of erythrocytes, less than 4% of which were parasitized, was added to subconfluent cultures of human umbilical vein endothelial cells and incubated at 37°C for 90" with gentle rocking. Afterwards, the adherent cells were washed, fixed, and stained.

Major Findings:

Individual endothelial cells bound as many as 100 infected erythrocytes. Although only a few per cent of the erythrocytes in the initial red blood cell suspension contained trophozoites and schizonts, nearly all (> 93%) of the bound erythrocytes contained these mature forms, thus representing a minimum 25-fold enrichment of these mature forms on the endothelial cell membrane. Comparable results were obtained with both infected human and Aotus erythrocytes. Only a subpopulation of endothelial cells seemed capable of binding infected RBCs although all of the vascular endothelial cells, regardless of their ability to bind infected RBCs, stained brightly for factor VIII antigen, a specific endothelial cell marker.

The binding of RBCs to cultured endothelial cells was specific as demonstrated by three observations. First, only infected RBCs bound to cultured endothelial cells; uninfected human and Aotus RBC suspensions demonstrated no binding, suggesting that parasite induced changes in the RBC membrane are necessary for adhesion. Second, infected RBCs did not bind to human foreskin fibroblasts or bovine aortic endothelial cells cultured under identical conditions. Third, only RBCs infected with mature asexual parasites were capable of binding; viable ring forms did not bind to cultured endothelial cells. Thus, binding was parasite stage specific in vitro as it is in vivo.

Scanning EM demonstrated numerous knobs (K) on the membranes of the cultured infected erythrocytes and transmission electron microscopy demonstrated that the knobs were the points of attachment between infected RBCs and cultured endothelial cells. Therefore, the gross mechanisms of in vivo and in vitro binding appeared to be the same.

Heat inactivated serum from an immune Aotus monkey known to contain antibodies directed against the knobs completely abolished binding whereas preimmune serum from the same monkey had no effect on binding. These results suggested that antibodies directed againt the knob ligand are capable of blocking attachment. In addition, these results are consistant with the hypothesis that antibodies directed against the knob ligand may play an important role in maintaining the immune state.

Significance to Biomedical Research

The results of this study demonstrate that Plasmodium falciparum infected erythrocytes attach to a subpopulation of endothelial cells via a parasite-associated determinant located in the knob of the infected erythrocyte membrane. This *in vitro* binding assay should aid in the isolation and characterization of the knob ligand, a potentially important immunogen for malaria prophylaxis.

Future Course of the Project:

1. Isolate the antibodies responsible for inhibition of binding. Examine the specificity of such antibodies as determined by their ability to block the attachment of various strains of Plasmodium falciparum.

2. Production of a monoclonal antibody capable of blocking attachment.

3. Immunoprecipitation of the surface labeled knob ligand.

4. Biochemical characterization of knob ligand.

5. Compare the ability of K^+ and K^- infected erythrocytes to bind to endothelial cells.

Pathophysiology of Fibrosis in Immune Disorders:

Project Description:

We are interested in the mechanisms of fibroblast activation in cell mediated immune disorders. We have chosen the prominent dermal fibrosis observed in patients with chronic graft versus host disease as a prototype and have utilized the mixed lymphocyte reaction as an *in vitro* method of generating factors capable of stimulating fibroblast proliferation and product biosynthesis.

Specifically, we intend to characterize the factors produced by human mixed lymphocyte reactions which are capable of stimulating fibroblast proliferation and collagen biosynthesis. The cell of origin, kinetics of secretion, modes of modulation, and biochemical characterization are targeted goals of this project. We intend to clearly distinguish these factors from others produced by activated mononuclear cells, e.g. T-cell growth factor and fibroblast chemotactic factor (LDCF-F) on a functional and molecular level.

Project Update:

Attention has been primarily focused on the functional and biochemical characterization of the fibroblast proliferation factors found in the secondary MLR supernatants. Gel filtration in the presence of non-ionic detergents has shown that the previously identified 120,000 dalton molecule is a complex between albumin dimers and a 15,000 MW molecule. When the 15,000 MW species was analyzed by preparative IEF, three charge species with pI's of 7.0, 6.2 and 5.4, respectively, were observed. In view of the similarity in size and charge between MLR fibroblast proliferation factors and human interleukin I, we employed several strategies to explore in more detail the relationship between these molecules. The following were observed: first, thymocyte proliferation activity, a functional measure of IL1 and fibroblast proliferation activity co-purified after a three-step purification protocol resulting in a 200 fold increase in specific activity; second, when the fractions obtained from the last step in the purification protocol, viz., IEF, were assayed for both thymocyte and fibroblast proliferation activities, nearly identical activity profiles were obtained; third, the three charge species were shown to induce the production of IL2 by a cloned murine T lymphoma line, LBRM-331A5, an additional functional property of IL1; fourth, partially purified human and murine IL1 both stimulate fibroblast proliferation. Other studies showed that human and murine IL2 did not stimulate fibroblast proliferation. Moreover, no IL2 activity was found in the partially purified preparations of MLR fibroblast proliferation factors.

Significance to Biomedical Science:

These studies suggest that IL1 is a fibroblast proliferation factor and thereby may have important regulatory functions with respect to non-lymphoid as well as lymphoid cells. Taken together with other studies showing that IL1 is a pyrogen and stimulates prostaglandin and collagenase release by synovial cells, these results suggest that IL1 may be an important inflammatory mediator and, in particular, may be in part responsible for the fibrosis observed in many chronic inflammatory states. Of additional interest is the finding that in vitro measurement of fibroblast proliferation is a convenient functional measure of only IL1 and not of IL2.

Studies of the TSTA of the Guinea Pig L_2C Leukemia:

The L_2C lymphatic leukemia of inbred strain 2 guinea pigs arose about 25 years ago in an old female strain 2 guinea pig. Studies in our laboratory starting 8 years ago have shown that this leukemia cell is of B cell origin and has surface IgM and C3 receptors. There are several variants of this leukemia; one variant exists which lacks Ia antigens (an antigen of the major histocompatibility complex of the guinea pig). Our laboratory has demonstrated that, as measured by immunization protection tests in inbred strain 2 guinea pigs, the L_2C cell has a powerful tumor specific transplantation antigen (TSTA). However, in the Ia negative variant of the L_2C cell, this TSTA is not immunogenic. Our previous studies suggested that the TSTA is not related to the surface IgM molecule.

The main goal of the present study is to identify the chemical nature of the TSTA of the L_2C leukemia cell. A 3M KCl extract of the L_2C cell contains the TSTA as measured by immunization protection tests in syngeneic strain 2 animals. Immunization with as little as 100 μg of this material per animal confers protection against a lethal challenge of L_2C cells. Fractionation of the KCl extract on Sephadex G-200, DEAE cellulose and CM cellulose indicate that the immunogenic molecule has a M.W. of less than 20,000 and is basic. The material is very resistant to heat and extremes of pH but is destroyed by treatment with trypsin, neuramnidase and periodate. These properties suggest that the TSTA is a low M.W. glycoprotein. Attempts to further fractionate the material by polyacrylamide gel electrophoresis were unsuccessful, none of the fractions eluted from these gels were immunogenic.

Most recent studies have used CM cellulose followed by G-50 chromatography. Several immunogenic regions from G-50 chromatography have been obtained and are now being currently investigated. In addition, Biogel chromatography on fractions obtained from CM Cellulose demonstrated a single broad peak of protein, using several different Biogel beads; the second half of these peaks consistently contains the immunogenic material as determined by immunization projections tests. We have also determined that the immunogenic fraction is not bound to a Con A column. When the KCL extract was extracted with organic solvents the active principle remained in the aqueous phase. Polyacrylamide gel analysis of several of active crude fractions showed up to 15 bands in these fractions. Recently, 10 fractions of the KCL extract were obtained by isotachypheresis - none of these 10 fractions were immunogenic. Progress in the chemical identification of the TSTA of the L_2C cell has been slow because each individual assay takes 7-8 weeks to complete (_in vivo_ protection being the final end point).

Significance to Biomedical Research and the Program of the Institute:

The properties of the TSTA of the L_2C leukemia cell are highly unusual and differ from the properties of most other TSTA described by other investigators. Most other TSTA have a M.W. greater than 40,000 daltons and are sensitive to heat.

The fact that this TSTA is on a malignant B lymphocyte is of particular interest; once the L_2C TSTA is more completely identified one could look for a similar TSTA in several different human B cell leukemias. Another possibility is that this TSTA could represent a marker for a particular stage of B cell differentiation present on only a very small number of normal B cells.

In addition since Ia^+ and Ia^- variants of the L_2C leukemia exist, these cells have proven useful in other programs for screening of monoclonal antibodies for Ia activity.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-AI-00035-06 LI

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Specificity in Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator:	John K. Inman	Sr. Investigator	LI, NIAID
Other Investigators:	Bruce Merchant	Chief, IB, BBP	BOB, FDA
	Chester Schott	Postdoctoral Fell.	BOB, FDA
	Charles DeLisi	Sr. Investigator	LTB, NCI
	H. Hethcote	Visiting Sci.	LTB, NCI
	R. Schwartz	Sr. Investigator	LI, NIAID
	D. Hansburg	Res. Associate	LI, NIAID; MB, NCI
	Harm Snippe	Asst. Prof. Micro.	State U. of Utrecht, The Netherlands

COOPERATING UNITS (if any)
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LAB/BRANCH
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SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The principal aim of this project is to test the hypothesis of general multispecificity for antibody combining regions and other types of receptors. Receptor sites should be capable of binding an occasional disparately structured ligand with an association constant large enough potentially to affect biological function. Radiolabeled antibodies or receptor-bearing molecules will be screened for multispecific binding to many, large haptens each bound to a separate affinity column matrix. Large haptenic reagents have been synthesized for this purpose using methods of peptide synthesis. Monoclonal antibodies from myelomas and hybridomas are being studied first. Multispecific bindings will be studied further by quantitative affinity chromatography.

Collaborative studies bearing on issues of specificity in immune responses have been and are being carried out; they deal with molecular interpretations of cellular mechanisms in immune responses. Chemical methods such as heteroligation and use of heterobifunctional reagents are employed in preparing specially designed antigenic materials, test reagents, and polyfunctional probes.

Project Description

Objectives

The overall objective of the project is to gain more complete understanding of the molecular basis for specificity in immune responses. The binding properties of antibodies and other receptor-bearing molecules are to be studied and related to the behavior of immune systems. Specific objectives are the following:

1. To assess the generality and frequency of multispecific binding by individual antibody combining regions by means of screening experiments employing many diverse, structurally unrelated haptens.
2. To develop methodology required for such screening tests including the synthesis of numerous, large haptenic reagents.
3. To use the screen in studying the multispecific character of other types of receptors and to employ observed cross-reactions in a general approach to affinity separations of receptors.
4. To employ methods and reagents developed in this project in collaborative studies dealing with specificity in immune responses.

Major Findings

The most important advance of the past year was the successful development of the micro-affinity chromatography binding test system that will be the principal technique employed in studies of multispecificity and fine specificity of antibodies and other soluble receptors. Work was completed on every phase and component of the method, and the assembled affinity column setup was used in determining correctly the single-site equilibrium association constant of a bivalent monoclonal antibody. Screening this immunoglobulin (MOPC 460) with a small panel of dye-ligands was begun, and several new specificities were found.

Accomplishments in developing the separate parts of the affinity chromatographic test system are listed and briefly described below. The binding assay is performed by slowly passing a radiolabeled sample of antibody or other ligand-binding protein through a small (usually 1-ml) column bed of an adsorbent that bears covalently bound hapten. Either buffer or buffer containing soluble hapten is employed for elution. Elution volume and the radioactivity profile are carefully measured.

1. Selection of adsorbent matrix: Early trials with DNP-bound polyacrylamide gels and the DNP-binding myeloma protein, MOPC 460, failed to give useful elution profiles: The non-porous beads apparently lacked sufficient interacting surface, so that no retention was observed; variable and eratic penetration (trapping?) of antibody molecules was observed with the most porous bead form available. De-sulfated, crosslinked agarose beads (Sepharose CL-4B, Pharmacia), on the other hand, gave excellent results and is now routinely being used. Suspensions of these beads can be stored in the frozen state and thawed repeatedly without physical or functional damage; this fact will be a tremendous advantage in the long-term storage and use of a large panel of specific adsorbents.

2. Initial functionalization of the support: The method that I published in 1975 for introducing carboxyl and amino groups into the synthetic polysaccharide, Ficoll, worked very well for preparing the aminoethyl-carbamylmethyl (AECM) derivative of Sepharose CL-4B. A simplified procedure was developed.

3. Addition of spacing structure: Several reagents were designed especially for adding hydrophilic β-alanine residues to amine supports for increasing the spacing of a terminal structure from the polymer matrix. The syntheses were worked out for N-protected, active esters of β-alanine and β-alanyl-β-alanine. When used in appropriate excess, these new compounds were found to combine with all support amino groups, and the protecting group (ethylsulfonylethoxycarbonyl) was thereafter rapidly removed with dilute solutions of NaOH.

4. Thiolation of supports through terminal amino groups was accomplished through the use of Carlsson's reagent (SPDP) or by the use of dithiobis-(succinimidyl propionate), followed in each case by dithiothreitol reduction. The latter method was developed in this study; each approach gives the simple, uncharged N-3-mercaptopropionyl derivative. This form of sulfhydryl agarose has proven to be an excellent substrate for attachment of haptens (as indicated below).

5. Attachment of haptens through formation of disulfide or thioether bonds was studied. Solutions to the problem of thiol-disulfide interchanges during the formation of disulfide linkages were found; yet, disulfide-linked haptens were not completely stable. Thioether linkages were found to be completely satisfactory in this regard. Alkylation of sulfhydryl groups with haloacetyl functions (e.g., 2-chloroacetyl) was chosen. Very high efficiencies of coupling (92 - 94%) were obtained in preparing adsorbents of quite low hapten densities, thereby allowing both economy and precision in haptenations.

6. Radiolabeling of immunoglobulins: Protein 460 and normal rabbit IgG have been effectively labeled with high specific activity, tritiated succinimidyl propionate from Amersham. By employing a set of conditions adopted in this study, from 35 to 58% of the labeling species can be bound to 1-mg samples of immunoglobulin. An improved immunoadsorption procedure for isolating protein 460 was designed and carried out.

7. Apparatus: Custom-designed (JKI) glass micro-columns (made by Wheaton Glass Co.) were used in conjunction with miniature, drop-counting fraction collectors (LKB). Fractions, in toto, were mixed with scintillation cocktail and counted, thus obviating the extra labor of aliquoting samples. A technique was developed for delivering fluids to open micro-columns at constant, controllable rates via capillary siphons.

8. Quantitative treatment of results: One of the most significant developments of this past year resulted from my collaboration with Dr. Charles DeLisi and Dr. Herbert Hethcote of the Laboratory of Theoretical Biology, NCI. I was able to translate their new theory of column chromatography involving chemical reactions (bindings) and non-equilibrium processes into an empirical expression that relates single-site, equilibrium binding constants with the experimental

A collaborative study with Drs. Chester Schott and Bruce Merchant (BOB/FDA) has been published that reported on genetic differences in immune memory in inbred mice to the TI-2 antigen, DNP-Ficoll. Direct and indirect, splenic plaque-forming cell responses were measured in mice following secondary challenge with this antigen. Strong IgM memory, but no IgG memory, was observed in SJL/J, AKR/J and C58/J mice. Strong IgM and relatively strong IgG memory responses were seen in the C57/J strain. C57BL/6N, C57BR/cdJ and A/HeJ mice were unable to mount either type of memory response under an identical immunization schedule. It was interesting to note that all of the first three strains (giving strong IgM and essentially no IgG memory responses) have a propensity to develop lymphoreticular neoplasias.

Finally, it may be mentioned that I have collaborated with Drs. Ronald Schwartz and Daniel Hansburg (LI/NIAID) on matters concerning chemical techniques (heterologiations, peptide synthesis, use of special protecting groups, etc.) in their studies on specificity relationships in T-cell stimulation using cytochrome c peptide variants. For details, see Dr. Schwartz's report.

Significance to Biomedical Research and the Program of the Institute

An essential characteristic of many immune mechanisms is the specificity of action which is directed by definitive chemical structures. An understanding of specificity in immune systems rests first upon an adequate knowledge of the scope and nature of specificity at the level of single receptor-determinant interactions. Therefore, a principal aim of this project is to continue working on the first systematic and general exploration of the phenomenon of multispecific binding. Because of the diversity and availability of antibodies, these receptor-bearing molecules provide ideal models for such studies. Receptor multispecificity may play a fundamental role in drug action and side effects and in toxicological reactions.

Cooperative studies have proven the value of large haptenic reagents and other synthetic or semi-synthetic antigenic components in exploring specificity as it is manifested in more complex, multireceptor-determinant interactions. Reagents or reactions developed for the explicit requirements of this project have interesting applications in other basic studies in immunology such as those aimed at elucidating the mechanisms of B- and T-cell specific activation. Techniques for heteroligation that are employed and further developed in these studies will have many important applications in areas such as manipulation of immune responses and in the preparation of clinically useful test materials as, for example, specifically sensitized red cells and ELISA reagents.

Future Course of the Project

Details of the binding measurements have been worked out, and the systematic testing of monoclonal immunoglobulins will soon commence. A principal source of the latter will be hybridoma antibodies donated by other laboratories. Synthesis of large haptenic reagents will continue on a more-or-less routine basis in order to enlarge the repertoire of the screening program.

The future course of the project will be directed toward the following parameters of my system. Thus, it was possible, from a group of 7 affinity profiles from runs with various bound and soluble hapten concentrations, to calculate the correct association constant of protein 460 for the DNP hapten. The theory allows for the fact that both mono- and bivalent bindings contribute to the observed retention. I devised a computational method for simultaneously obtaining two empirical coefficients and the binding constant from a quadratic equation and, at the same time, optimizing the use of data when more than the minimal amount is collected. These relationships and the general theory allow a full understanding of behavior of affinity columns, so that various approaches to screening can be rationally designed and evaluated.

Preliminary screening of protein 460 with small a dye-ligand panel revealed three newly recognized specificities: 4-nitrobenzo-2-oxa-1,3-diazole ($K \sim 10^{4.3}$), Procion Red HE3B ($K \sim 10^{3.2}$) and Cibacron Blue F3G-A ($K \sim 10^{3.3}$). I am reporting the above developments in affinity chromatographic techniques in two invited papers at the coming 4th International Symposium on Affinity Chromatography (June 22 - 26, 1981) at Veldhoven, The Netherlands.

A new approach to hapten synthesis was initiated based on the use of the 2-chloroacetyl group as a protecting group and as an activated function. Chloroacetyl derivatives of L-lysine have been prepared, accordingly, for use in synthesizing the next series of screening haptens. Dr. Shukla has continued to work in my lab as a part-time guest worker this past year and has been able to purify and characterize some of his triazine-based haptenic reagents. These compounds can now be used in the screening program for disparate structure specificities.

N-Haloacetyl β-alanyl peptide active esters have been synthesized as general heteroligating reagents and for use in preparing special haptenic compounds. The feasibility of converting chloroacetylated haptens into more reactive iodoacetyl analogues by an interchange reaction was successfully demonstrated. The iodo analogues are more suitable for crosslinking, heteroligation, or haptenation steps involving cells and other fragile structures.

In the area of collaborative studies, three more publications (see list below) have appeared in the literature (since the last annual report) that resulted from my collaboration with Dr. Bruce Merchant (BOB/FDA) and Dr. Harm Snippe (State University at Utrecht, The Netherlands). These papers deal with several aspects of the X-linked, B-cell defect in CBA/N mice and the similarly defective F_1 male progeny of CBA/N X C3H/HeN hybrids. Two of these papers report studies showing that uptake or binding of haptenated TI-2 antigens is normal; that is, the CBA/N defect is not attributable to an inability of defective B cells to effectively bind these antigens either in vivo or in vitro. Defective cells can take up antigen and "convey" it to functional B cells in cell transfer systems, resulting in their stimulation. Studies on the hapten-specific blockade of CBA/N X C3H/HeN F_1 B-cell responses in a cell transfer system revealed interesting differences in blockading effects as compared with studies of simple in vivo immune responses. However, these different effects applied only to secondary immune responses in the cell transfer studies.

specific objectives:

1. To assess the occurrence frequency of interactions of antibodies with disparately structured haptens in various binding energy ranges and to compare results with expectations from theoretical models.

2. To test other ligand-binding proteins (e.g., lectins, transport proteins and soluble receptors) for multispecific behavior.

3. To use the testing facility for locating some diverse specificities of antibodies, etc. for use in affinity purification and in functional studies in various systems.

4. To use the testing facility to develop disparate specificity profiles as clonal markers in studies of clonal expression control.

5. To study thermodynamic and structural aspects of multispecific interactions for gaining a better understanding of multimodal binding.

6. To develop theoretical models for explaining selectivity, specificity and reliability in systems involving components with multispecific binding sites.

Collaborative studies will continue where chemical methodology, developed and used in the above studies, is applied in various areas of basic and applied immunological research.

Publications

Inman, J.K.: Peptide synthesis with minimal protection of side-chain functions. In Gross, E., and Meienhofer, J. (Eds.): The Peptides-Analysis, Synthesis and Biology. New York, Academic Press, 1981, pp. 253-302.

Snippe, H., Merchant, B., Lizzio, E.F., and Inman, J.K.: Localization of ^{125}I-labeled DNP-Ficoll on lymphoid cells from the normal and defective hybrid progeny of CBA/N mice. Int. Arch. Allergy Appl. Immunol. 62: 168-178, 1980.

Snippe, H., Merchant, B., Lizzio, E., and Inman, J.K.: Functional antigen binding by the defective B cells of CBA/N x C3H/HeN F_1 male mice. J. Immunogenetics 7: 409-420, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-AI-00037-14 LI

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunogenetics of Mouse Immunoglobulins and Genetic Control of Antibody Response.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator:	Rose Lieberman	Sr. Investigator	LI, NIAID
Other Investigators:	W.E. Paul	Chief	LI, NIAID
	J. Kung	Visit. Fellow	LI, NIAID
	M. Potter	Sr. Investigator	LCB, NCI
	S. Rudikoff	Sr. Investigator	LCB, NCI

COOPERATING UNITS (if any)

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Laboratory of Immunology

SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our research program encompasses studies of allotypes and idiotypes of mouse immunoglobulins and their role as genetic markers. These markers are used in the investigation of the genetic organization of the Ig gene complexes and in the regulation of their expression in the immune response. Idiotypic determinants T15 and C3, expressed on anti-phosphorylcholine antibodies and U61, expressed on anti-levan antibodies, were used as Igh-V region structural markers in inbred and wild mice. T15 and C3 behave like allelic markers in inbred mice but in wild mice were frequently found to have recombined. Wild mice of species that were distantly related to Mus musculus frequently exhibit none of the known allotypes but sometimes express T15 and U61 but not C3. More closely related wild mouse species exhibited the same allotypic markers as inbred mice and also expressed T15, C3 and U61 markers. However, the associations of allotypes and idiotypes seen in inbred mice were not observed in wild mice. These findings suggest that recombination may have occurred frequently in evolution or possibly that idiotypic and allotypic expression are regulated by other genes.

A. Igh-C and Igh-V Markers in Wild Mice

In inbred mice, genetic and structural studies show that the C3 idiotypic determinant is associated with anti-phosphorylcholine (PC) antibodies of C57BL mice and with a PC-binding myeloma protein of C.B20 mice, a strain which bears C57BL Igh genes on a BALB/c genetic background. These markers behave like products of allelic genes in inbred mice. The T15 idiotypic determinant is associated with anti-PC antibodies of BALB/c mice and with PC binding myeloma proteins of BALB/c mice. In inbred strains four phenotypes have been found. $C3^+T15^-$ is associated with the b and j Igh-C haplotypes; $C3^-T15^+$ with the a, and $C3^-T15^-$ with the c, d, e, f, and g haplotypes. The $C3^+T15^+$ phenotype is observed in BAB-14 mice, which possesses a recombinant Igh haplotype derived from both C57BL and BALB/c elements. Wild mice distantly related to *Mus musculus* were usually $C3^-T15^-$ or $C3^-T15^+$. Species more closely related to *Mus musculus* were phenotypically $C3^+T15^+$, $C3^-T15^+$ or, rarely, $C3^-T15^-$. Frequently, many of the mice in the latter group appeared allotypically homozygous and suggested frequent recombination of C3 and T15 Igh-V markers or, possibly, regulatory control of the expression in these markers. In addition, in wild mice there was no concordance of allotype and the specific allelic forms of Pc antibodies observed in inbred strains.

Species of wild mice both closely and distantly related to *Mus musculus* produce anti β(2→6) fructosan binding antibodies on immunization with levan. Only the closely related species produced anti β(2→1) fructosan (inulin) binding antibodies. An idiotype (A48) associated with the anti β(2→6) antibodies of BALB/c mice and with levan binding myeloma protein was not found in any of the wild mice tested. However, the U61 cross reactive idiotype, associated with BALB/c anti-inulin antibodies and with a BALB/c inulin-binding myeloma protein was frequently present in antibodies of the more closely related species of wild mice. The linkage of the anti-inulin idiotype to specific allotypes observed in inbred strains was not found in the expression of this response of wild mice.

B. Progeny Studies of Wild Mice (Eastern Maryland Shore) That Are Phenotypically $Igh\text{-}c^{e\text{ or }f}$ and which Fail to Make Anti-PC

All inbred mice that have been immunized with the R36 strain of pneumococcus or with PC-hemocyanin conjugates respond by producing anti-PC antibodies. A large group of wild mice derived from the same deme on the Eastern shore of Maryland that were similarly immunized failed to produce anti-PC antibodies and were phenotypically $Igh\text{-}C^e$, $T15^-$ or $Igh^f\text{-}C$, $T15^-$. These mice were bred to BALB/c mice ($Igh\text{-}C^a$ $T15^+$) and their F2 progeny are being studied for their anti PC response and the segregation patterns of the a, e, and f allotypes and of the idiotypes $T15^+$ and $T15^-$.

C. Studies of Allelic Forms of Anti-PC Antibodies in Progeny of Wild and Inbred Mice

The phenotype $Igh\text{-}C^b$, $C3^+$, $T15^+$ is presumed to be an allelic form of anti-PC antibodies in BAB14 mice. This phenotype has been also found to be prevalent among Mus musculus Czech I and Czech II wild mice as well as in other wild mouse species. In inbred strains other than BAB14, T15 and C3 behave like alleles and are linked to allotypes $IghC^a$ and $IghC^b$ respectively. Czech I mice of $Igh\text{-}C^a$ or $Igh\text{-}C^e$ allotypes have been mated to DBA/2 which are phenotypically $Igh\text{-}C^c$, $T15^- C3^-$. Backcross and F2 progeny will be examined for their Ig phenotypes and the segregation patterns of the allotypic markers a, c, and e and of the idiotypic markers $C3^+$, $C3^-$, $T15^+$ and $T15^-$.

Future Potential

Ig genetic markers (allotypes and idiotypes) have proven to be very useful in studies of the complexities of the cellular network and its role in conferring immunity. For the most part conventional antisera prepared with specific antigen binding myeloma proteins have been used to identify the multiple antigenic specificities associated with idiotypes. The introduction of techniques to produce monoclonal antibodies now permits the examination of the individual idiotypic specificities (idiotopes) of B cells and antibodies and also allows the assessment of the role of T cells in expression of given idiotopes.

Our laboratory is presently engaged in preparing monoclonal antibodies to idiotopes associated with anti levan and anti-phosphorylcholine antibodies. These monoclonal antibodies will be employed in cellular studies to help evaluate mechanisms of cellular interaction in eliciting immunity.

PUBLICIATIONS

Lieberman, R., Rudikoff, S., Humphrey, W., Jr., and Potter, M. Allelic forms of anti-phosphorylcholine antibodies. J. Immunol. 126:172, 1981.

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Lieberman, R., Potter, M., Rudikoff, S., Humphrey, W., Jr., and D'Hostelaere, L. Allelic forms of anti-phosphorylcholine antibodies; their expression in inbred and wild mice. In Immunoglobulin Idiotypes and Their Expressions (Eds. C.A. Janeway, Jr., E. Sercarz, and H. Wizell) Academic Press, N.Y. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00036-16 LI

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunoglobulin Genetics: Regulation of Gene Expression and Lymphoid Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	K. Bernstein	Research Assoc.	LI, NIAID
	N. Francis	Staff Fellow/ Guest Worker	LI, NIAID

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LAB/BRANCH

Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9	6	3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Overall goals of this project are to define the nature of genes which code for, or otherwise affect the structure of immunoglobulins (Igs) and to understand the processes which regulate Ig-gene expression and lead to synthesis of highly specific antibody molecules. We prepare immunological reagents that detect structural differences between genetically controlled polymorphic forms of rabbit Igs (Ig allotypes). We use immunological, chemical and molecular genetic approaches to define the Ig-structural differences and to learn more about the chromosomal organization of Ig genes, the regulated expression of antibodies, and antigen-specific receptors on lymphocytes. Inheritance patterns and the levels of regulation of phenotypic expression of alleles and chain types are investigated by breeding experiments, by studies of ontogeny and differentiation of cells of the rabbit immune system and by direct molecular genetic analyses. We use recombinant DNA technology to examine genome organization by isolation of rabbit Ig genes and to prepare cDNA copies of Ig mRNA. Characterized DNA probes will permit us to identify and analyze Ig variable and constant region DNA sequences as well as adjacent sequences and to characterize nuclear RNA precursors and their processing steps in the production of Ig mRNA.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The objectives of this research program are to define in molecular terms, the organization and regulated expression of rabbit immunoglobulin genes, the levels of regulation of lymphoid cell differentiation, and the mechanisms that lead to the synthesis of highly specific antibody molecules and antigen-specific lymphocyte cell surface receptors. We seek a unified picture of the levels at which expression of Ig genes can be affected, and a detailed molecular description of the mechanisms that lead to the regulation.

Methods Employed:

For these studies, we prepare and characterize antisera that detect structural differences between genetically controlled polymorphic forms of rabbit Igs (Ig allotypes) and between classes and subclasses, types and substypes of their polypeptide chains. We use immunological and chemical approaches to define the structural differences recognized by our antisera. We conduct both classical and molecular genetic experiments to learn more about the chromosomal organization of genetic information for the structure and regulated expression of Ig molecules. Problems which until recently have been approached at the levels of serology and classical Mendelian genetics are now amenable to direct molecular genetic analysis. We have cloned rabbit genomic DNA, prepared mRNA from cells producing rabbit Igs and cloned complementary DNA copies of the Ig mRNAs so that we can examine the organization of rabbit Ig genes and identify the nuclear RNA precursors and processing steps in the production of Ig mRNA. We breed and maintain colonies of rabbits with defined Ig allotypes and pedigrees for use in these studies. The rabbits and reagents we produce are made available to numerous other investigators at the NIH and are shipped in cooperation with the NIAID Research Resources Branch to investigators in laboratories throughout the U.S. and around the world.

Major Findings:

Breeding Experiments. We have developed a colony of rabbits by selective breeding that are homozygous and heterozygous for the Basilea trait (bas) for defective production of immunoglobulins with kappa-type light chains. We have prepared IgG and light chains from homozygous bas mutant rabbits and raised antisera by immunization of b^4b^6 and b^4b^9 rabbits with the IgG that allow us to type homozygous and heterozygous animals carrying the bas trait. We have demonstrated that the antisera detect a light chain produced by the bas mutant rabbit that is probably of kappa type. Its expression is regulated at the b (kappa) locus and the trait segregates in our breeding studies as an allele (or pseudoallele) of the b allotypes b4, b5 and b6. The b9 allotype has not been included in the breeding studies because the bas rabbits produce a kappa type light chain that is b9-like. The b9-like kappa chain may be the product of a gene distinct from that for the bas kappa light chain. The bas gene may represent a duplicated κ constant region that is closely linked to b9, or a b9-like gene, and normally unexpressed or poorly expressed. The b9, b9-like and bas may be isotypic forms of kappa light chains. If this is so, our breeding studies may allow us to detect recombinations that might be predicted to occasionally occur between bas and the kappa allotypes b4, b5 or b6. However, because some of the kappa chain genes may be unexpressed or poorly expressed, our molecular genetic approaches using DNA probes may reveal more about the organization of the genes for kappa chains in these mutant as well as normal rabbits.

Cooperation between our laboratory and those of Drs. Kindt, Mandy, Hamers, Dray, Knight and Hanly has led to exchanges of serum samples and correlations of typing data to yield new information about the IgM and IgA allotypes present in the pedigrees of three recombinant rabbits in which genes for V_H (a,x,y) and C_γ (d,e) appear to have undergone recombination. The recombinant haplotypes have provided support for the hypothesis that some allotypic determinants on IgM molecules are present only when a specific V_Ha allotype is expressed with a specific C_μ allotype. The μ mRNA that we are currently using for cDNA synthesis is from our recombinant haplotype.

Structure and allotypes of rabbit immunoglobulins

We published the sequence of most of the constant region of rabbit kappa type light chains of b5 allotype (Chersi, Alexander and Mage, 1980). The cDNA we are currently analyzing was also prepared from mRNAs for b5 kappa type light chains. This sequence will thus allow us to compare the DNA and protein sequences.

Fragments from the variable and constant regions of rabbit lambda type light chains were sequenced and assigned position numbers in comparison with known sequences of lambda chains of man and mouse. Lambda light chains were prepared from the IgG of fully suppressed $\underline{b^5b^5}$ homozygous rabbits as well as from a λ-rich IgG fraction from b9 rabbits. For 82 positions of constant region compared, the rabbit λ light chain sequence is about as homologous to mouse λ (76% homologous to λ 1, 68% to λ 2) as mouse λ 1 is to λ 2 (about 64%). The striking homologies between rabbit and mouse lambda chain sequences are comparable to those between the rabbit kappa chain allotypes b4, b5 and b9 (62-80%). The λ sequence data also suggest that there are at least two different J-coding sequences in the rabbit (Chersi and Mage, 1981). Additional structural studies utilizing light chains prepared from the IgG of homozygous bas rabbits are in progress.

Ontogeny, gene expression, and lymphoid cell differentiation

Our recent investigations of rabbit lymphoid cell differentiation have included studies of allelic exclusion and allelic gene expression in pre-B cells and B cells of normal and allotype-suppressed heterozygous rabbits. We are currently investigating the nature of the defect in bas mutant rabbits which leads to their production of serum Igs that are almost entirely of lambda light chain type. Affinity purified fluorescent antibodies specific for the μ heavy chains of rabbit IgM, for the heavy chain variable region allotypes (V_Ha) and for the kappa light chain allotypes b4, b5 and b9 were used to examine pre-B and B cells for expression of these markers. The majority of surface μ negative, cytoplasmic μ positive, large (12-18 μm) rabbit pre-B cells from a^2a^3 heterozygotes have detectable a2 or a3 but not both. Small (6-12 μm) pre-B cells of the same phenotype are also found. Pre-B cells expressing a2 or a3 are found in approximately equal numbers in heterozygotes whereas B lymphocytes expressing a3 appear to undergo preferential clonal expansion early in development. Rabbit bone marrow and blood also contain a population of myeloid cells that stain for both a2 and a3 when from heterozygotes. The frequencies of this cell type are not changed by prolonged incubation at 37°C, but are readily reduced after cell suspensions are treated with low pH buffer. These cells appear to bear high avidity Fc receptors for serum Ig and may account for some of the reported observations of double-allotype expressing B lymphocytes in heterozygotes.

A small proportion of rabbit pre-B cells (∿ 10%) express kappa chain allotypes. The pre-B cells that produce detectable light chains are relatively small (6-12 μm). In heterozygotes, individual cells express one or the other allelic kappa chain allotype but not both. Based on our studies and others on man and mouse, we have proposed that B cells with allelically excluded V_Ha and C_{κ}b allotypes are derived from the small transitional μ^+, κ^+, pre-B cells. In bone marrow from b^5b^9 heterozygotes, pre-B cells and immature B lymphocytes expressing b9 were as numerous as those expressing b5. In contrast, cirulating B cells and bone marrow plasma cells expressing b5 outnumber b9 by 2:1 in adult b^5b^9 heterozygotes. Thus the "pecking order" appears to be established during the clonal expansions of B cells.

Because these and our previous studies showed that pre-B cells in rabbits are generally unaffected by mechanisms which regulate the expression of allelic b allotypes in heterozygotes (for example, the "pecking order" and allotype suppression), we hypothesized that products of kappa type genes might be found in the pre-B cells of Basilea rabbits. Indeed although the homozygous bas rabbits produce serum Igs that are almost entirely of lambda type, the marrow pre-B cells of newborn bas homozygotes stained with our affinity purified anti-b9 reagent but not with anti-b4 or anti-b5. The proportion of bas pre-B cells with detectable kappa type light chains was comparable to that found in the marrow of normal animals studied at the same time (8% vs. 12%). When heterozygous b^4b^{bas} rabbits were compared with b^4b^9 normals, both types of heterozygotes had equal numbers of $b4^+$ and $b9^+$ (or b9-like) pre-B cells. Although B cells with b4 predominate over b9 by 4:1 in adults, immature marrow B cells of b4 and b9 type were found in equal numbers. In contrast, there were significantly fewer b9-positive B cells in b^4b^{bas} compared to b^4b^5 heterozygotes (2% vs. 31% of μ^+ B cells). It thus appears that either the b9-like light chain produced by bas rabbits is not properly assembled into membrane IgM and inserted in the cell surface, or clones of B cells bearing the b9-like light chain do not expand because they fail to produce effective receptors for antigen or are suppressed by an active regulatory mechanism.

Molecular Genetic Studies

Rabbit mRNAs. We have prepared and are in the process of preparing total RNA and poly A^+ mRNAs from the spleens of 25 rabbits of defined heavy and light chain allotypes. In order to increase our yields of mRNAs, spleens are collected when rabbits are making elevated responses to bacterial vaccines or are infected with *Trypanosoma equiperdum*. We have shown that the trypanosome infection results in a marked elevation of serum IgM and that the spleens of such animals are a rich source of mRNAs for rabbit Igs especially IgM. The mRNAs have been shown to direct the cell free synthesis of Ig heavy and light chains in a messenger dependent reticulocyte lysate system. Proteins labeled with ^{3}H-Leu and ^{35}S- Met have been characterized electrophoretically on SDS-PAGE. Immunoprecipitations with specific anti-kappa (b) allotypes, anti-V_H, anti-Ig and anti-IgM reagents have demonstrated production of kappa light chains of specific allotype as well as μ and γ heavy chains. Spleen cells from several mRNA donors were also labeled in culture to allow study of the products of cellular synthesis and comparison with products of cell free synthesis. Cells were also cultured in the presence of the antibiotic Tunicamycin to inhibit glycosylation. Different electrophoretic mobilities of light and heavy chains observed for products of cellular and cell free systems are consonant with synthesis of cell free products with precursor (signal) peptides and non glycosylated heavy chains in the cell free system and by cells cultured with Tunicamycin. The total and poly -A$^+$ mRNAs have also been characterized on methyl mercury hydroxide gels and shown to be intact. Some mRNAs have been transferred to Northern paper for later use in identification of DNA probes. Sucrose gradient fractions of mRNA have been obtained that are highly enriched for messages for κ, γ, and μ chains. Having mRNA that is efficiently translated into rabbit heavy and light chains is an essential tool that is now being used for screening and characterization of cDNA clones and genomic sequences that may correspond to rabbit Ig H and L chains.

Rabbit DNAs. Total mRNA and mRNA fractions enriched for kappa, γ and μ messages have been and are being used for synthesis of cDNA. Double stranded, tailed cDNA from total mRNA has been inserted into plasmid pBR322 by the GC tailing method and transformed bacteria selected for tetracycline resistance and scored for ampicillin sensitivity. One set of individual transformed colonies (1,226) has been identified, picked and stored. Screening has been done to identify colonies carrying inserts of rabbit DNA sequences that hybridize strongly with labeled mRNA from which the cDNA was made. Selected positive colonies (78 to date) have been rescreened with labeled mRNAs from sucrose gradient fractions enriched for heavy and light chain messages and more than 30 show strong hybridization. Inserts of rabbit DNA sequences have also been identified in a number of the clones after plasmid DNA preparation, restriction enzyme digestion and gel electrophoresis. Inserts range in size from 350 bp or less up to approximately 3400 bp. We are using the technique of message selection to screen for cDNA inserts carrying rabbit DNA sequences for Ig heavy and light chains. Positive controls are mouse tumor and spleen mRNAS selected by appropriate mouse DNAs. Immunoprecipitations of the proteins synthesized by messages selected by the first six clones tested have been completed and are on radioautographic film now. Known controls have been shown to be positive.

Rabbit Genomic DNAs. Genomic DNA libraries in lambda bacteriophage Charon 4A constructed from rabbit sperm DNA and spleen DNA have been plated, DNAs lifted onto nitrocellulose filters and screened to date with mouse kappa DNA and J_H DNA.

Candidates for potential kappa positive phage have been picked, grown and phage prepared. Potential J-positive phage are currently being identified. Sperm have been collected from more than 25 different rabbits of a variety of a and b (heavy and light chain) allotypes. High molecular weight DNAs have been prepared and digestions with various restriction enzymes, gel electrophoresis and Southern blots made. These blots provide material needed in conjunction with the recombinant DNA clone screening.

Mouse δ Chain Gene Expression

A cDNA clone, pδ54J, containing a major portion of the mouse δ chain constant region derived from mouse myeloma TEPC 1017, has been used to identify the δ RNAs present in plasmacytoms by hybridization of RNAs separated by electrophoresis on methyl mercury hydroxide agarose gels; one major and three minor RNA species have been observed in TEPC 1017 RNA (Mushinski et al., 1980). Further hybridization studies (Fitzmaurice et al., in press) utilizing the cDNA clone and fragments of the δ gene have indicated that the four δ chain RNAs found in IgD-secreting plasmacytomas and the two found in normal spleens are encoded by combinations of different exons. Different δ gene segments code for alternate 3' termini, and these RNAs probably represent mRNAs for the different forms of heavy chain found in secreted and membrane-bound IgD.

The processing pathways for generating mature mRNAs for secreted and membrane-bound δ chain are being studied in the IgD-secreting plasmacytomas, normal spleen, and B cell tumors including BCL_1-5 and BAL 17. The possibility that μ and δ RNA are derived from the same primary transcript is being examined in these latter two cell lines.

Significance to Biomedical Research and the Program of the Institute

Immunogenetics continues to be at the forefront of our understanding of the nature of structural and regulatory genes in complex mammalian systems. As we increase our knowledge of the genetics and evolution of Ig genes and of the chemistry of Ig proteins and the DNAs that code for and regulate their production, the Ig system becomes one of the most valuable models for investigation and understanding of the functioning of complex genetic regions on chromosomes. These systems are providing new knowledge of the molecular basis for cellular differentiation in higher organisms. The inter-relationships of the cell surface antigenic systems controlled by the major histocompatibility locus and the Ig gene systems are becoming increasingly apparent as knowledge of the two systems increases. The increased knowledge of Ig genetics provides models of direct relevance to understanding the evolution and genetic organization of cell surface antigenic systems. Understanding the chemistry, genetics and evolution of Igs, the factors that control their genetic expression and the differentiation pathways of the cells which produce them is of broad fundamental interest.

Proposed Course of the Project

Most of the projects described are in progress and continuing. In order to further characterize the kappa type light chains produced by bas rabbits, we are attempting to elevate their production by hyperimmunization of bas homozygotes. We are attempting to produce enough high titer anti-bas antiserum to allow affinity purification, absorption and fluorochrome labeling for studies of bas rabbit marrow pre-B and B cells. These studies should allow us to determine immunochemically and at the cellular level whether bas and b9-like chains are distinct and products of different cells. The molecular genetic studies are at the stage where we expect to be characterizing rabbit DNA inserts by restriction enzyme mapping and DNA sequencing. The characterized DNAs will then be used as probes to identify and study structural genes for rabbit Ig H and L chains in differentiated and non-differentiated cells and to identify and characterize nuclear RNAs and the processing intermediates of Ig mRNAs. For these studies we already have collected rabbit sperm and various organs including B-cell depleted thymocytes as sources of DNA, and we are exploring the use of cultured rabbit lymphocytes that may produce normal or "latent" gene products. Methyl mercury hydroxide agarose gel electrophoretic analyses and hybridization studies of mouse δ chain RNAs currently in progress in our laboratory provide a model system for our anticipated studies of the transcription and processing of rabbit immunoglobulin RNAs. As outlined above, we expect that molecular genetic approaches will provide new insights into the molecular basis for phenomena observed in the rabbit model including the bas mutation, "hidden gene" expression, imbalances in quantitative expression of "allelic" genes, alleleic exclusion and cis expression of V_H and C_H genetic information.

Appendix

Contract number N01 AI 82565-maintenance and Breeding of Rabbits of Known Genotype and Their Use in Immunological Studies.

The objectives and methods are related to those outlined above. The contract has continued to provide outstanding support through production and maintenance of allotype-defined rabbits, immunizations, bleedings, and various short-term and long-term projects involving breeding, immunizations and suppression. A procedure for collection of rabbit sperm from sperm DNA donors is in operation. Rabbits and reagents produced and supplied through this contract were made available to numerous other investigators at the NIH and were shipped to laboratories throughout the U.S. and around the world.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER
		Z01-AI-00147-06 LI

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Mechanism of Activation of Thymus-Derived Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other Investigators: John Inman, Ph.D., Sr. Investigator, LI/NIAID; Ettore Appella, Ph.D., Sr. Investigator, LCB/NCI; Louis Matis, M.D., Clinical Associate, LI/NIAID; Ellen Heber-Katz, Ph.D., Postdoctoral Fellow, LI/NIAID; Benjamin Sredni, Ph.D., Visiting Scientist, LI/NIAID; Daniel Hansburg, M.D., Ph.D, Research Associate, LI/NIAID; Lawrence Samelson, M.D., Research Associate, LI/NIAID; Steven Hedrick, Ph.D., Postdoctoral Fellow, LI/NIAID; Charles Hannum, Graduate Student, Dept. of Biochemistry and Molecular Biology, Northwestern University; Emanuel Margoliash, Chairman, Dept. of Biochemistry and Molecular Biology, Northwestern University

COOPERATING UNITS (if any)

LCB/NCI, NIH; Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, Illinois.

LAB/BRANCH

Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.5	7.5	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project focuses on the mechanism and function of immune response (Ir) genes in the activation of thymus-derived (T)-lymphocytes using a secondary T-cell proliferation assay and the recently developed technology of T-lymphocyte cloning. With these techniques we have demonstrated that Ir gene complementation stems from the requirement for two separately encoded polypeptide chains to form a single Ia molecule involved in antigen-presentation. Furthermore, we have accumulated evidence that two separate sites exist on the antigen molecule, pigeon cytochrome c fragment 81-104, and that the recognition of only one of these sites appears to be under Ir gene control. These results support presentational models of Ir gene function. Finally, we have isolated clones of antigen-specific T lymphocytes and shown that some of them also possess allo reactivity. This result demonstrates conclusively that these two T cell repertoires are over-lapping sets.

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(Rev. 10-76)

The relationship between those T lymphocytes which recognize alloantigens and those which recognize all other foreign antigens has puzzled immunologists for many years. The former are present at such a high frequency that most investigators have felt that there must be some overlap between the two populations. During the past year we have demonstrated that this is indeed the case by isolating and characterizing clones of T lymphocytes which express both specificities. Using the soft agar cloning technique developed in our laboratory, we isolated antigen-specific T cell clones from B10.A mice immunized with dinitrophenylated-ovalbumin (DNP-OVA). These cells were then screened for their ability to respond to alloantigens on spleen cells from mice of 8 independent haplotypes in the absence of DNP-OVA. Of 38 colonies examined, 8 showed reactivity toward one of the spleen cell populations. This frequency, 21%, is extremely high considering that only 8 haplotypes were tested out of a potential of 100 or so which may exist in the entire *Mus musculus* species. This result suggests that all antigen-specific T cell clones possess an alloreactive specificity since the 30 antigen-specific clones which did not show any alloreaction against the spleen cells tested would probably react against one of the 92 or so untested haplotypes. Another interesting aspect of the data was the fact that the 8 alloreactivities detected were not random. Four of the colonies were stimulated by B10.S spleen cells, 2 by B10.D2 spleen cells, 1 by B10.RIII spleen cells, 1 by B10.M spleen cells and none by spleen cells from the 4 other strains tested. These results suggest that there is some relationship between the receptor on the cell which recognizes DNP-OVA and the receptor which recognizes the alloantigen. This linkage could be at the DNA level with co-expression of particular pairs of receptors in any given cell, or it could represent cross-reactive recognition by a single receptor on the cell surface. Future studies will be directed toward elucidating this relationship.

In a continuation of our studies on the biologic basis of immune response (*Ir*) gene complementation, we have studied the genetic control of the T-cell proliferative response to the protein antigen, pigeon cytochrome *c*. Similar to what was found for the synthetic polypeptide, GLΦ, two Ir genes were required in the B10.A mouse to generate a response, one mapping in the *I-A*^{k} subregion, the other in the *I-E*^{k} subregion. Presentation experiments showed that both Ir gene products must be expressed in the same antigen-presenting cell in order to stimulate a T cell response. Furthermore, the *I-E* subregion gene product could be donated by almost any strain expressing the serologic marker Ia.7. This result supported our previous model that complementation stems from the combination of an *I-A* encoded *β* polypeptide chain and an *I-E* encoded *α* polypeptide chain to form the two chain functional Ia antigen that is required for presentation of antigens like pigeon cytochrome *c*. An interesting new aspect to come out of these experiments was the finding that an Ia.7 bearing strain, B10.PL, did not complement for pigeon cytochrome *c* responsiveness or presentation. Biochemical and serological studies, carried out for us by Dr. Pat Jones of Stanford University and Dr. Donal Murphy of Yale University, demonstrated that the critical molecule necessary for function, $\beta_{AE}^{\kappa}\,\alpha_{E}^{\mu}$, was present in [B10.A(4R) x B10.PL]F_1 cells but in greatly reduced amounts (approximately 1/8). With this knowledge in mind, we isolated long term T cell lines and T cell colonies specific for pigeon cytochrome *c*. These allowed us to optimize the antigen presentation experiments and to demonstrate that the [B10.A(4R) x B10.PL]F_1 presenting cells possessed a quantitative defect in their ability to present pigeon cytochrome C. Thus, what was originally viewed as an exception to the model of gene complementation was now shown to extend the model by demonstrating the critical concentration dependence of the complementing Ia molecule for antigen presentation.

In a continuation of our studies on the characterization of antigenic determinants recognized in a T lymphocyte proliferative response, we found experimental evidence to support an antigen presentation model of Ir gene function. One of the essential characteristics of an antigen-presentation model is that there must exist two separate sites on the antigen involved in stimulating the response. One of these sites is recognized by the receptor on the T lymphocyte, the other is recognized by the Ir gene product of the macrophage. Other models require only a single site on the antigen for the T cell receptor to recognize. When B10.A mice were immunized with the 81-104 cyanogen bromide cleavage fragment of pigeon cytochrome c and their T cells stimulated in vitro with cytochrome c fragments 81-104 from several other species, a pattern of cross-reactions was observed which allowed one to map certain critical residues involved in stimulating the response. The pattern conclusively identified the glutamine residue at position 100 and demonstrated that a serine could substitute for the glutamine without loss of antigenicity. If the same B10.A mice were immunized with an acetimidyl chemical derivative of pigeon cytochrome c fragment 81-104 (Am-pigeon 81-104) a different population of T cells appeared to be primed since the T cells could not be stimulated with the native pigeon 81-104. Similarly, B10.A T cells primed to the native pigeon 81-104 were poorly cross-reactive to the Am-pigeon 81-104. The surprising result was obtained when the critical residues involved in the Am pigeon 81-104 antigen were mapped using acetimidyl derivatives of the species variants of cytochrome c fragments 81-104. The pattern of cross-reactions was almost identical to that observed for the native pigeon 81-104, suggesting that the same glutamine = serine residue was involved in stimulating the response. Thus, two poorly cross-reacting immune responses appeared to share recognition of one antigenic site, but the fact that they were poorly cross-reactive demanded that a second antigenic site, designated the Lys/AmLys site, must also be recognized. A study of the immune response genes involved in the T cell proliferation to the native and Am-pigeon 81-104 antigens suggested that the Ir genes were also the same because the strain distributions of responsiveness were identical and a monoclonal antibody directed against the same Ia molecule blocked both responses. Finally, the specificity of the immune response to Am pigeon 81-104 in the B10.A(5R) was examined to determine the effect of changing Ir gene(s). In this strain the native pigeon 81-104 still gave no stimulation but the pattern of cross-reactions with Am-derivatized fragments from other species was completely different from that seen in the B10.A. This result suggested that the cross-reactive patterns were functions of Ir gene constraints on specificity of the immune response and not merely secondary consequences of chemical properties of the cytochrome c antigens that influenced, for example, their uptake or degradation. Thus, the receptor specificity which was used in common in the B10.A for the recognition of both native and Am-pigeon 81-104, appeared to be the one that was influenced by Ir genes. Therefore, we feel these data can be interpreted as evidence for two receptor specificities for the antigen, only one of which is under Ir gene control, a prediction of an antigen presentation model of Ir gene function.

Significance to Biomedical Research

Our attempts to understand the mechanism of T-lymphocyte activation and Ir gene control of this response are of fundamental importance to a basic understanding of how the immune system functions. Since this system plays a critical role in the body's defense mechanisms against infectious diseases, tumors and transplanted tissues, achieving our goal would hopefully provide major insights into how to manipulate the immune system for the benefit of the patient.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER
		Z01-AI-00040-07 LI

PERIOD COVERED

October 1 , 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Control of Immunocompetent Cell Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7	4	3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

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SUMMARY OF WORK (200 words or less - underline keywords)

The long range goals of this project are to determine the role of I-region associated (Ia) antigens in the regulation of immunocompetent cell interactions and to determine the mechanism of action and site of expression of the major histocompatibility complex (MHC)-linked immune response (Ir) genes. During the past year we have focused our studies on three areas: 1) We have developed the technology to produce cloned lines of I-region restricted, antigen-specific as well as alloreactive guinea pig T cells. We have combined the use of these cloned lines with monoclonal anti-Ia antibodies and demonstrated that individual antigen-specific clones recognize antigen in association with distinct epitopes on Ia molecules. 2) Evidence for the importance of Ia antigens in T cell activation was derived from studies of the syngeneic mixed leukocyte reaction (SMLR) in which we demonstrated that Ia antigens in the absence of foreign antigens activate multiple antigen-specific T cell clones and that the SMLR represents antigen-independent polyclonal activation of antigen-specific T cells mediated through their receptors for self-Ia. 3) We have also developed the immunochemical techniques for the analysis of macrophage antigen processing and have identified a critically important pool of antigen present on the macrophage surface.

I. The Production of Antigen-Specific and Alloreactive T Cell Clones

Culture fluids from antigen or mitogen stimulated lymphoid cells contain growth factors that permit the long term growth of T cells. We have adapted for use with guinea pig cells the procedures used for the production of murine antigen-specific T cell clones in soft agar and have successfuly produced both antigen-specific MHC-restricted proliferating clones as well as alloreactive clones which proliferate specifically on stimulation with the appropriate Ia-bearing macrophage. About 80% of our initial picks from soft agar expand in liquid culture and a very high percentage (95-100%) of the expansion cultures are antigen or alloantigen specific. We have been able to maintain most of the clones for several months in the continuous presence of T cell growth factor, antigen, and syngeneic stimulator cells and our oldest clones are now greater than one year old. We can easily cryopreserve and recover the clones with complete preservation of antigen specificity.

The availability of relatively large numbers of homogeneous populations of T cells should greatly facilitate the analysis of the process of T cell activation, the role of Ia antigens in T cell recognition, and the isolation of the antigen specific receptor from the T cell surface. We have already begun to use both the antigen specific and the alloreactive clones in a variety of studies aimed at an analysis of these questions. We have investigated in detail the cellular and molecular requirements for optimal proliferative responses of the alloreactive clones. We noted that optimal proliferative responses of the clones were observed in cultures containing purified macrophages and T cell growth factors; however, in some experiments proliferative responses were also detected in the presence of allogeneic peritoneal exudate cells (PEC) in the absence of factor. We demonstrated that the T cell component of the stimulator cell population was necessary for this response because it produced growth factors endogenously in the cultures. Thus, two signals, allogeneic Ia antigens and growth factors, were required for optimal proliferative responses of these clones; either signal by itself was insufficient to induce a response. Furthermore, macrophage associated Ia antigen was more efficient than B cell associated Ia in the induction of a response. The requirement for allogeneic Ia antigen was not absolute as the colonies could also be expanded when the cultures were supplemented with irradiated syngeneic PEC and T cell mitogens. The effect of the mitogen was mediated via the T cells in the irradiated PEC as removal of the T cells from these PEC markedly reduced the response. Thus, a second mechanism of activation of these alloreactive T cells appears to be mediated via a non-specific signal presumably from T cells which can promote the proliferation of alloreactive T cell clones in the absence of allogeneic Ia antigen.

II. The Combined Use of Monoclonal Antibodies and Cloned T Cell Lines As Probes of Macrophage Ia Antigen Function.

Anti-Ia antibodies have proven to be extremely useful tools in defining the relationships between Ia antigens and Ir gene product function. Over the past two years we have studied the effects of monoclonal anti-Ia antibodies on T cell proliferation and observed several differences between the results obtained with the monoclonals and those previously observed with conventional alloantibodies to Ia. Conventional anti-Ia sera produced a marked inhibition of the *in vitro* antigen induced proliferative response to all antigens tested as well as producing potent inhibition of the MLR. In contrast, monoclonal anti-Ia reagents produced a selective pattern of inhibition in that the responses to some, but not all antigens were inhibited; monoclonals produced only modest inhibition of the MLR even at very high concentrations. This result suggested to us that a single monoclonal antibody might react with different parts of an Ia molecule and might participate in the presentation of certain antigen or antigen fragments, but not others. One difficulty with the approach we have used is that we have studied the effects of the monoclonals on populations of T cells from primed animals that would be composed of multiple subpopulations of cells directed to multiple Ia determinants seen in association with a complex protein antigen.

The use of the T cell cloning technology described above resolves most of these problems. Thus, a single T cell clone should be reactive to antigen in association with only a single determinant or epitope on the Ia molecule and should be inhibited by the appropriate monoclonal antibody. When a panel of anti-Ia monoclonal reagents were used to inhibit the proliferative responses of a large panel of both antigen specific and alloreactive T cell clones, the proliferative respones of the individual T cell clones were often completely inhibited by some monoclonal anti-Ia antibodies but not by others. This result is consistent with the hypothesis that monoclonal anti-Ia antibodies inhibit T cell proliferation by masking individual Ia epitopes and that individual antigen specific T cell colonies recognize antigen in association with such distinct epitopes on Ia molecules; alloreactive clones similarly are specific for distinct epitopes on allogeneic Ia molecules.

These results add a new dimension of fine specificity both to the T cell's recognition process and to the role of Ia molecules in antigen presentation. Furthermore, these results may have important implications for the relationship between Ia antigens, Ir genes, and disease susceptibility. It has been postulated that some of the frequently noted associations between disease susceptibility and HLA antigens in man may be associations between disease susceptibility and distinct epitopes on HLA antigens. Determination of such disease-MHC epitope associations could prove to be of great practical and theoretical value by allowing more accurate detection of individuals at highest risk for developing certain diseases.

III The Syngeneic Mixed Leukocyte Reaction (SMLR) and Antigen-Specific T Cell Activation

The SMLR represents a T cell proliferative response that occurs upon *in vitro* stimulation by syngeneic non-T cells. In the guinea pig, the primary stimulator of the SMLR is an Ia-positive PEC and the reaction can be specifically inhibited by anti-Ia sera directed to the stimulator cells. To further elucidate the role of Ia antigens in the process of T cell activation in the SMLR, we have used both positive and negative selection procedures to correlate the recognition of self-Ia molecules on the stimulator cell with the recognition of conventional foreign antigens by the same T cell population. We have shown that negative selection of SMLR primed T cells abrogates their subsequent responses to foreign antigens, but has little effect on their response to allogeneic Ia. A direct relationship between the SMLR reactive and the foreign antigen reactive T cell population was seen in positive selection experiments in which (2 X 13)F_1 T cells were primed in the SMLR to stimulator PEC from one or the other parental strain. In subsequent primary and secondary assays of T cell proliferation, such SMLR primed T cells demonstrated a pronounced preference for antigen in association with stimulator cells of the parent used in the primary culture. These results strongly suggest that self-Ia molecules on the stimulator cell population can activate multiple antigen specific T cell clones and that the SMLR represents antigen independent polyclonal activation of antigen-specific T cells through their receptors for self-Ia. Further detailed study of this population of T cells with high affinity receptors for Ia as well as antigen specific receptors is of critical importance as diminished or suppressed SMLR responses have been observed in several disease states in both man and experimental animals.

IV. The Expression of Immunologically Relevant Antigen on the Surface of the Antigen-Presenting Macrophage

The failure of antibody to soluble protein antigens to inhibit T cell proliferation to antigen-pulsed macrophages has raised the possibility that immunologically relevant antigen is not expressed on the macrophage surface. We have previously shown that when macrophages were pulsed with soluble protein antigens, aged overnight, and then modified with trinitrobenzenesulfonic acid (TNBS) prior to addition to primed T cells, significant inhibition of the T cell proliferative response was observed when the cultures were performed in the presence of anti-TNP antibody. The inhibition was specific in that antigens which could not readily be TNP-derivatized were not inhibited when treated in the above manner and then cultured in the presence of anti-TNP antibody. Our working hypothesis is that when antigen is present on the surface of the antigen presenting cell it is modified with TNBS with subsequent binding of sufficient anti-TNP antibody to both the antigen and TNP-labeled membrane proteins resulting in capping and shedding of the antigen from the cell surface with subsequent inhibition of T cell proliferation.

We have used this model for an initial biochemical approach to macrophage antigen handling or processing. Guinea pig PEC were pulsed with the ^{125}I-labeled terpolymer, L-glutamic acid, L-lysine, L-tyrosine (GLT), and then derivatized with TNBS either immediately after pulsing (fresh) or 24 hours after pulsing (aged). Detergent extracts of these cells were analyzed with anti-GLT and anti-TNP sera in a quantitative radioimmunoassay. Fresh GLT pulsed macrophages contained a pool of intracellular GLT as well as a distinct pool of cell surface GLT. In contrast, aged GLT pulsed cells lacked intracellular GLT, but expressed GLT on the cell surface. Functional studies performed in parallel indicated that anti-TNP antibody was only capable of inhibiting the response to aged-TNBS-treated macrophages, but not the response to fresh-TNBS-treated cells. These results demonstrate that immunologically relevant antigen is expressed on the macrophage surface and raises the possibility that processing of antigen occurs during the period of time immediately after pulse exposure of the macrophage to antigen during which time a significant fraction is found in an intracellular rather than cell surface site. The involvement of Ir gene products in this step of antigen processing is presently under study.

V. The Induction of Specific Transplantation Tolerance by Splenic Allografts

It has previously been shown that spleen allografts between MHC incompatible inbred rats survived spontaneously in certain strain combinations. The recipients were then specifically tolerant of subsequent skin and pancreas allografts. The mechanism permitting spleen allograft survival in rats has thus far not been determined. We have extended these studies to the inbred guinea pig because strain 2 and 13 only differ in the I-region of the MHC and because of the ready availability of sensitive in vitro assays of T lymphocyte function.

Ninety per cent of spleen grafts 13 to 2 survived spontaneously throughout the lifetime of the hosts whereas the spleen grafts 2 to 13 were rejected within 3 weeks. Serial MLR studies on lymphocytes from strain 2 recipients with surviving spleen transplants revealed an early markedly suppressed host anti-donor MLR and concomitantly a significant host anti-"self" MLR. The latter was shown to be mediated by chimeric donor cells. These in vitro findings were paralleled clinically with a high mortality from graft versus host disease. Animals that survived this initial period of GVH continued to be permanently MLR suppressed against the donor strain, yet with an absence of the above described reactivity against "self." Such animals accepted donor type skin allografts indefinitely and rejected third party skin allografts. This type of specific transplantation tolerance was adoptively transferable to normal non-irradiated strain 2 guinea pigs, but could not be transferred by sera. Studies are now in progress to further characterize and isolate the suppressor cell responsible for the induction and maintenance of tolerance in this model.

Significance for Biomedical Research:

The major histocompatibility complex plays a critical role in the regulation of several aspects of the immune response. Studies in man as well as in several experimental animal systems have demonstrated that genes within the MHC control disease susceptibility. Our studies are designed to determine the mechanism of action and site of expression of MHC-linked Ir genes. Although we have elected to study experimental models where simple protein antigens are used, the results we will obtain should have direct applicability and should give important insights to the analysis of the role of Ir genes in the control of disease susceptibility in man.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00038-09 LI

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure and Activity Studies on Immunologically Important Cells and Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other Investigators:	Dr. M. Iwata	Visiting Fellow	LI, NIAID
	Dr. J. Wantyghem	Visiting Fellow	LI, NIAID
	Dr. B. Mathieson	Sr. Staff Fellow	LMI, NIAID
	Ms. B.J. Fowlkes	Biologist	LMI, NIAID
	Ms. S. Sharrow	Chemist	I, NCI
	Dr. M. Kaliner	Head, Aller. Dis. Sec.	LCI, NIAID

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The early biochemical events which result in lymphocyte stimulation by extracellular signals are being sought. These signals include the action of non-physiological agents, such as mitogenic lectins, and also soluble factors which transfer information between cells. The earliest enzymatic event detected thus far is the activation of membrane associated transmethylases. This is followed by increased calcium ion flux, activation of phospholipase A2, arachidonic acid release and increased activity in the lipoxygenase pathway.

To facilitate these and other studies, we are also seeking to identify and fractionate subpopulations of lymphoid cells. In addition to our ongoing experiments, using specific lectins to recognize cell surface glycan differentiation markers, we are evaluating the use of neoglycoproteins and polysaccharides to identify specific carbohydrate receptors on subsets of lymphoid cells.

In the past year this project has focussed primarily on the lymphocyte plasma membrane. The structures in this membrane receive all external signals to the cell (such as control of proliferation or differentiation) and identify the cell (such as thru MHC antigens and differentiation markers) to adjacent cells and organs. Two continuing major areas of study were expanded this year and are reported separately below.

Biochemical Events in Transmembrane Signaling and Lymphocyte Activation

The prime objective of this project is to determine the earlier (first few minutes) biochemical events and pathways which are important in the activation of B and T lymphocytes. We have previously elucidated a series of biochemical steps in the activation of lymphocytes by mitogenic lectins, soluble factors, and anti-immunoglobulin. These include sequential methylation of phosphatidylethanolamine to form lecithin, and its degradation to arachidonic acid and lysolecithin along with increased flux of calcium ions through the cytoplasmic membrane. From this time course of these reactions it was unclear whether or not the two enzymic reactions were linked.

Our current studies have shown that specific methylation inhibitors block both reactions in parallel dose-response curves. The removal of calcium ions from the activation mixture has no effect on the methylation reaction, but blocks activation of phospholipase A2, lecithin breakdown, and subsequent DNA synthesis. This suggests that the order is methylation of phospholipids, increased flux of calcium, activation of phospholipase A2, and the release of arachidonic acid.

While methylation and calcium flux appear to be required for phospholipase A2 activation, these events are not sufficient for full stimulation. Apparently phospholipase A2 is bound to a 40,000 dalton glycoprotein inhibitor, which may be identical to lipomodulin. One of the events in lectin induced lymphocyte activation may be the binding of this glycoprotein inhibitor by the lectin, which would result in the unblocking of phospholipase A2. Lipomodulin was first described by Axelrod's laboratory in other systems, and a hybridoma antibody has been raised against this phospholipase inhibitor. We have used this antibody (anti-LM) to study the activation pathway. Anti-LM does not stimulate DNA synthesis by itself. However, when added to cells stimulated with mitogenic doses of Con A, it totally blocks exhanced DNA synthesis and increases cell death. There is also evidence to suggest that anti-LM may synergize with submitogenic doses of Con A to stimulate DNA synthesis, but the scatter in this data is discouraging.

The role of released arachidonic acid in this activation pathway is also being evaluated in collaboration with Dr. M. Kaliner (LCI, NIAID). There are two major pathways for further metabolism of this fatty acid. The cyclooxygenase pathway leading to prostaglandins and thromboxanes and the lipoxygenase pathway leading to the various hydroxy and hydroperoxy derivatives of eicosatetraeonic acid. Specific inhibitors exist for the cyclooxygenase pathways. We have found that they do not specifically inhibit lectin induced DNA synthesis. Unfortunately a similar set of specific inhibitors does not exist for the lipoxygenase pathways. However, inhibitors which block both pathways also abrogate lectin stimulated DNA synthesis. These results suggest that the lipoxygenase pathway of arachidonic acid metabolism may be the transmittal route for cell activation.

The transmethylation of phospholipids is an early and controlling event for the evocation of several cellular phenomena. The elucidation of subsequent biochemical steps will further our understanding of the control mechanisms of the activation of immunocompetent cells and may be generalizable to other biological systems. These pathways will continue to be studied in detail by use of radioactive metabolites, specific inhibitors and enzyme assays.

Fractionation of Lymphoid Cell Subsets

Our collaborative studies with scientists in LMI and others, identifying and fractionating subsets of lymphoid cells by using lectins which specifically bind to cell surface glycan differentiation markers, have proven successful and are continuing. This year the main focus of effort occurred in LMI and the details of the study are reported under Project Number Z01-AI00136-09 LMI.

In addition, we have initiated a complementary series of experiments. There have been a few reports which indicate that lymphoid cells possess cell surface carbohydrate binding proteins (lectins). If such lectins differentially exist on subsets of cells, and are specific in the carbohydrates they recognize, then they have the potential of being of prime importance in immunology. These cell surface lectins would be excellent candidates for active participation in cell-cell recognition, homing, and intercellular signaling. Further, they may serve as a new set of differentiation markers for lymphoid cells.

Our initial studies, in collaboration with Dr. Monsigny (CNRS, Orleans, France), have used fluoresceinated neoglycoproteins (synthetic glycoproteins with a selected carbohydrate moiety) as probes to determine the occurrence of sugar specific cell surface lectins on murine lymphoid cells. The initial results showed that more B lymphocytes bind these fluorescent neoglycoproteins than T lymphocytes. The binding was found to be specific and inhibitable by the proper sugar. For example, 90% of B cells bind mannosyl neoglycoproteins while only 5% of T cells can bind this probe. α-N-Acetyl-Galactosamine probes are bound by 80% of B cells and by 25% of T cells. Murine thymocytes bind little or none of the tested neoglycoproteins. Attempts to correlate the binding of specific sugar probes with conventional cell markers or other probes have been inconclusive thus far. This is due to non-specific binding of our neoglycoproteins when coupled with the available red fluorescent dyes. These studies will be repeated with neoglycoproteins and antibodies to conventional markers when the red labeling problems have been minimized.

The finding that fluorescein labeled neoglycoproteins specifically bind to subsets of murine lymphocytes suggested the possibility of specifically agglutinating subsets of lymphoid cells by binding to polysaccharides. For this purpose, about two dozen homo- and heteropolysaccharides were prepared or obtained. Unfortunately, most commercially available material required repurification. The results identified 6 polysaccharides which agglutinated murine lymphoid cells. The proportion of cells agglutinated by each polysaccharide differed and ranged from 5% to 25%. In several cases, the cells removed by this specific agglutination could be recovered by washing the aggregate with specific mono- or disaccharides. The feasibility of using this approach for the rapid fractionation of functional subsets of murine lymphoid cells is being evaluated.

The classification of new differentiation markers, both cell surface glycans recognized by lectins and cell surface lectins recognizing external carbohydrate structures, will aid our understanding of the different types of lymphoid cells and their roles in the immune response. The development of simple lymphocyte fraction procedures based upon these differentiation markers will greatly facilitate experiments on the interactions between lymphoid cells.

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1981 Annual Report
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Summary Report
Laboratory of Immunoregulation
October 1, 1980 through September 30, 1981

Anthony S. Fauci, M.D.
Chief, Laboratory of Immunoregulation, NIAID
Deputy Clinical Director, NIAID

Mechanisms of Activation of Human B Lymphocytes

Over the past year, this laboratory has successfully developed the first truly antigen-induced, antigen-specific system to measure human B cell responses against soluble antigens. The antigens used were keyhole limpet hemocyanin (KLH) and tetanus toxoid (TT). The success of the system was based on the realization that antigen-reactive B cells are present in the circulation for only a limited time following in vivo immunization. A system was set up whereby normal volunteers and certain patient groups were immunized in vivo with the soluble antigen KLH and/or TT. A number of interesting and important observations were made. By a series of pilot experiments, it was learned that the optimal concentration of antigen that was needed to stimulate B cells was orders of magnitude less than the optimal concentration needed to trigger T cell blastogenesis. In fact, the most important observation was that the concentrations of antigen that actually triggered T cell blastogenesis were directly suppressive to the B cells in question. Another observation of extreme importance in the successful development of this system was the fact that associated with the antigen-specific induction of antibody responses in vitro was a necessarily concomitant polyclonal Ig response. This interfered with the ability to precisely examine antigen-specific responses alone. By altering the culture conditions, we were able to dissociate this otherwise invariable overlap between polyclonal Ig production and specific antibody production in response to the antigens in question. Therefore, under well defined culture conditions, we were able to demonstrate the first truly antigen-induced, antigen-specific triggering of circulating human peripheral blood B lymphocytes. Mastering of this antigen-specific system for human B cell activation has allowed us to precisely delineate mechanisms of B cell activation and immunoregulation never before possible in the non-specific systems previously used. In this regard, the kinetics of activation of the circulating B cell repertoire were described. The multiple subsets of B cells and their sequential appearance in the circulation following in vivo immunization were delineated as were the isotype switches which occurred following immunization. The system was shown to be absolutely dependent on the presence of monocytes and T cells and the precise monocyte and T cell interactions and mechanisms of immunoregulation were described in detail in subsequent studies. Based on the ability to specifically trigger antigen-reactive cells in vitro, a hapten-carrier system for the induction of hapten-specific B cell responses was successfully developed. Utilizing this system the relationship between carrier-specific T cells and hapten-specific B cells were described in human circulating lymphocytes.

This system of antigen-induced, antigen-specific activation of human B lymphocytes will now be used to dissect out the precise mechanisms of activation and immunoregulation of human B cell function in normal individuals and in a number of disease states characterized by abnormalities of immune function. (Lane, Volkman, Falkoff, and Fauci, LIR/NIAID).

Immunoregulation of Human B Lymphocyte Responses.

Utilizing the newly developed antigen-induced, antigen-specific in vitro activation of human B cells as described above, a number of studies directed at the delineation of the antigen-induced suppression of B cell function were undertaken and were extremely productive. It was found that at high antigen concentrations introduced into culture, the antigen in question reacted with the antigen specific T cells resulting in a substantial T cell blastogenic response. In addition to this blastogenic response, nonspecific T cell factors were elaborated which triggered multiple clones of B cells to secrete polyclonal Ig. In other words, high concentrations of antigen such as KLH in a KLH-immune individual resulted in an amplification of the response in vitro which led to the triggering of multiple clones of B cells. However, this same antigen concentration which triggered polyclonal Ig responses resulted in no immunoglobulin production of the antibody specificity related to the antigen in question. A number of studies definitively proved that the unresponsiveness to the specific antigen introduced in culture was due to a direct tolerization of the B cells in question and was not due to the induction of antigen-specific or even nonspecific suppressor T cells. This was demonstrated by preexposing various subsets of T cells as well as B cells with the antigen in question as well as by eliminating suppressor subsets of cells by the use of hybridoma antibodies and complement to specifically remove various functional subsets of T cells.

Thus, this was the first demonstration in the human system of direct antigen-induced, specific tolerance of B lymphocytes. In addition to B cell tolerance, the role of immunoregulatory T lymphocyte subsets were studied in detail with regard to the phenotype of the immunoregulatory T lymphocyte subsets as determined by monoclonal hybridoma antibodies against lymphocyte surface antigens. The gradation of irradiation sensitivity of immunoregulatory lymphocyte subsets in this system was delineated as well as the role of allogeneic effects in the antigen-specific B cell system. Allogeneic factors could replace T cells, but on the other hand, were responsible for allogeneic suppression of the system. The role of the monocyte was studied in detail and a number of interesting and potentially important results were found including: the absolute requirement for monocytes, the suppressive capability of monocytes, the role of the monocyte as presenting cell, the strict HLA-DR restrictions in monocyte-T cell interactions, the necessity for B cells to react with native antigen in association with the monocyte-T cell interactions necessary for optimal B cell triggering. In addition, the role of monocyte derived humoral factors in this complex monocyte-T-B cell interaction was studied. Furthermore, the immunoregulation of the system by hybridoma monoclonal antibodies directed against the antigen in question was studied and it was found that a complex idiotype-anti-idiotype interaction occurs upon the introduction of idiotype specific monoclonal antibody into the system. Another interesting finding related to the elimination of antigen-specific B cells by the conjugation of the toxic substance ricin to the antigen in question with subsequent specific binding of the antigen to the antigen-reactive B cells leading to their selective depletion. These studies have obvious clinical implications in attempts to eliminate autoreactive B lymphocytes. (Lane, Volkman, Falkoff, Kerhl, Gerrard, Peters, and Fauci, LIR/NIAID).

Pharmacologic Modulation of Human Immune Responses.

Corticosteroids are widely used in clinical medicine in the treatment of inflammatory and/or immune mediated diseases. This laboratory has been involved over the past several years in the delineation of the *in vivo* and *in vitro* effects of corticosteroids on human immune function. Over the past years, these studies have centered on the effects of corticosteroids on the activation and immunoregulation of human B cell function. A number of interesting observations were made including the selective suppression of chronically administered corticosteroids on human B cell function by a number of complex mechanisms including the selective enrichment in the circulation for suppressor T cells by the predominant depletion of helper T cells. This depletion was effected by a shift in the population of helper T cells to extravascular lymphoid compartments. In addition it was noted that corticosteroid treatment resulted in increased activation of T cells as well as the introduction into the circulation of an immature T cell subset.

A number of studies were performed on the precise mechanisms of cytotoxic agents on the activation and immunoregulation of human lymphocyte function associated with our study of a number of patient populations treated with these agents. It was found that cyclophosphamide had a highly selective effect on human B cell function with relative sparing of the T cell subsets at dosages of drug used in patients with immune mediated diseases. Of particular note was the fact the equivalent dose regimens of azathioprine therapy had no similar suppressive effect on the B cell function of our patient population. This observation is of particular relevance given the fact that cyclophosphamide has been found to be significantly more effective than azathioprine in the treatment of B cell mediated diseases. This observation lends a scientific explanation to a commonly observed clinical phenomenon. In addition, patients treated with cyclophosphamide were found to have markedly depressed natural killer (NK) function. Since intact NK function is believed to play a role in immune surveillance, particularly against lymphoproliferative diseases, this observation may help explain the emergence of lymphoid neoplasms in certain patient populations treated with cyclophosphamide. (Cupps, Lane, Gerrard, and Fauci, LIR/NIAID).

Generation, Characterization, and Mechanisms of Human Cytotoxic Cell Activity In Vitro.

Cytotoxic activity in man falls under a number of specific categories including cytotoxic T lymphocytes (CTL), natural killer cell (NK) and antibody dependent cytotoxicity (ADCC). Cytotoxic activity can be generated by a number of mechanisms including the mixed lymphocyte reaction (MLR). Over the past year, we have delineated several of the mechanisms of activation of the various cytotoxic lymphocyte activities. The cell types involved in the generation of cytotoxic activity in the MLR as well as the actual cytotoxic subpopulations of cells themselves were characterized by the expression of various cell surface phenotypes defined by monoclonal hybridoma antibodies. The molecular mechanisms operative in the induction and expression of cytotoxic cell activity particularly with regard to the role of the cyclic nucleotides as well as the inducers of cyclic nucleotides were delineated by a series of studies on NK and ADCC activity. In this regard, we described the first example of a primary immunodeficiency disease with an isolated defect in NK cells in two of our patients with Chediak-Higashi syndrome. This was shown to be truly a functional defect since the abnormality was not due to a lack of effector cells or an inability of effector cells to bind normally to target cells. (L. Moretta, A. Moretta, Ludwig Institute, Lausanne, Switzerland; Katz, University of Florida; Gerrard, Fauci, LIR/NIAID).

The Use of Hybridoma Antibodies to Delineate Activation and Immunoregulation of Human Lymphocytes.

The important advances associated with the hybridoma technology as described by Milstein and Kohler were utilized by this laboratory to establish a number of monoclonal hybridoma antibodies directed against human lymphocyte surface antigens. We produced and described in detail a number of hybridoma antibodies which defined a) cells with inducer T cell function for help and suppression; b) states of activation of lymphoid cells; c) expression of antigens following MLR activation; and d) clonal expansion of cells in certain lymphoproliferative states. These monoclonal antibodies have proven to be extremely useful not only in identification of various functional lymphocyte subsets in normal humans and in disease states, but have also been used to prepare highly purified populations of cell suspensions depleted of the respective lymphocyte subset utilizing the cytotoxic capability of the monoclonal antibody. In addition, a mouse-human heterohybridoma which is secreting pure monoclonal human anti-KLH antibody was established. The antibody has been shown to be mitogenic for lymphocytes from KLH immune individuals but not from KLH non-immune individuals suggesting a relevant and potentially extremely important idiotype-anti-idiotype interaction with the KLH immune cells. Moreover, the heterohybridoma which is producing pure human monoclonal anti-KLH antibody will serve as an excellent model for the production and perpetuation of hybridomas making pure human antibody against relevant microbial antigens. The potential clinical importance of this type of a system and the availability of such purified antibodies in large quantities is extremely exciting. (Lane, Butler, and Fauci, LIR/NIAID; Haynes, Duke University; L. Moretta and A. Moretta, Ludwig Institute, Lausanne).

Development of Soluble Antigen-Specific T Cell Clones.

We have successfully produced the first soluble antigen-specific T cell clones in man and have perpetuated these with several sub-clonings. They are truly clones by the strict definition and have thus far been demonstrated to be antigen-specific, genetically restricted by HLA-DR with regard to the absolute requirement for presenting cells, and are not alloreactive. The implications of developing antigen-specific T cell clones are enormous. In addition to providing truly "pure" T cell populations for functional assay, it provides a source of T cell determinants without contamination from other cell types and other T cell subsets.

Thus, the pressing question of the nature of the T cell receptor can now be approached as well as other questions of the relationship between the expression of surface antigens and functional capabilities. In addition, since these cells are fully characterized with regard to HLA and DR, a whole host of studies relating to the genetics of human immune reactivity can be approached from an entirely new and potentially productive vantage point.

Following a number of subclonings, we have characterized the KLH-specific human T cell clone according to surface antigen phenotype employing various receptor bindings and monoclonal antibodies directed against cell surface antigens. The T cell clone was shown to be OKT3+, OKT4- and OKT8+, thus expressing the cytotoxic/suppressor cell phenotype. In addition, we found it to bind to sheep red cells, express Ia antigens, lack Fc receptors for IgG and IgM. In addition, they express the "activation" antigen 4F2.

In several functional studies of co-culture of the clones with KLH immune B cells, it was shown that small numbers (10^3 cells) completely suppress the KLH-induced specific anti-KLH response in the presence of antigen without suppressing the polyclonal Ig response of the B cells in culture. As greater numbers of the suppressor clone were added to culture (10^4 and 10^5 cells), both antigen specific as well as polyclonal Ig responses were suppressed suggesting both a specific and non-specific component to the suppressor mechanism. (Volkman and Fauci, LIR/NIAID; Sredni and Schwartz, LI/NIAID).

Studies in the Idiopathic Hypereosinophilic Syndrome (HES).

A broad range of clinical and pathophysiologic studies were performed in a large number of patients with the HES. The precise mechanisms of organ system damage and dysfunction were delineated predominantly with regard to the endocardiomyopathy of the HES. The major basic protein of the eosinophil was demonstrated to be directly toxic to endothelial cells and likely accounts for the first insult to the endocardium. This was followed by a laying down of platelet thrombi with subsequent mural thrombosis and endocardial fibrosis leading to the restrictive cardiomyopathy. Although a portion of these studies were performed in an *in vitro* system, they are the first to strongly suggest the role of an eosinophil product in the direct damage to human tissue in the HES. In addition, *in vitro* stem cell studies delineated the heterogeneous levels of abnormalities in patients with HES ranging from primary aberrancies of eosinophil production to the regulation of eosinophilopoiesis. Furthermore, predictive parameters of the heart disease in HES as well as specific genetic profiles were described. Aggressive medical and surgical approaches to the heart disease as well as chemotherapeutic regimens aimed at the underlying eosinophilia have led to a dramatic improvement in prognosis of the HES. The study of these patients has allowed the possibility of studying several of the functional capabilities of the eosinophil ranging from those related to their enzyme content to others relating to the cytotoxic and other functional capabilities of the cells. (Harley and Fauci, LIR/NIAID; Gleich, Mayo Clinic; Bjornson, Tufts Medical Center).

Mechanisms of Abnormal Cellular Activation and Immunoregulation in Immune Mediated Diseases.

Utilizing the above mentioned systems of specific and non-specific triggering and immunoregulation of human B cells as well the delineation of immunoregulatory T cell and monocyte subsets, a series of studies were performed to delineate the abnormalities of immune function in a number of immune-mediated diseases. It was demonstrated that patients with Hashimoto's thyroiditis had abnormally high numbers of thyroglobulin (Tg)-reactive B cells in their circulating B cell repertoire. In addition, they were capable of being triggered by specific and/or non-specific stimuli to secrete anti-Tg antibody. This system will prove invaluable in examining the mechanisms of aberrant immune function in this disease. In addition, this laboratory has been involved in studies of abnormal cellular activation and immunoregulation in Sjogren's syndrome as well as in SLE for a number of years. Over the past year, a number of studies further delineating the mechanisms of hyperreactive B cells as well deficiency of immunoregulatory T cells were carried out. A group of patients with disseminated cutaneous leishmaniasis were studied and it was demonstrated that they expressed high numbers of polyclonally activated B cells most likely related to chronic stimulation with the parasite antigen. These observations may help explain polyclonal hypergammaglobulinemia seen in this and other parasitic diseases. Furthermore, it was demonstrated that patients with Chediak-Higashi syndrome had an isolated defect in NK cells which indeed is the first description in man of a primary immunodeficiency disease with such an isolated defect in killer cells. It was demonstrated that patients with dermatitis herpetiformis (DK) had abnormalities in RES function. Most importantly, in control experiments done together with study of the DH patients, it was demonstrated that normal individuals with the HLA-B8, DRw3 haplotype not only had abnormalities in RES function but actually expressed a number of immunoregulatory abnormalities such as a deficiency of a immunoregulatory T cell subset as well as increased numbers of B lymphocytes spontaneously secreting Ig. This demonstration of abnormalities of immune function in normal individuals related to a particular genetic haplotype has important implications in understanding the genetic predisposition to certain autoimmune and immune mediated diseases in man. (Lane and Fauci, LIR/NIAID; Katz, University of Florida, Gainsville, Florida; McGregor, Welsh National Medical School, Wales, UK; Lawley, Dermatology Branch/NCI; Frank, LCI/NIAID).

Clinical, Immunopathogenic and Therapeutic Studies in the Spectrum of Vasculitis.

The LIR is now prospectively studying the largest group of patients with the vasculitic syndromes of any center in the world. Clinical, pathophysiologic, immunopathogenic and therapeutic results obtained over the past 13 years have allowed us to design a revised categorization scheme for the vasculitides which has now reached world wide acceptance. We have instituted aggressive chemotherapeutic regimens in several, formerly universally fatal diseases such as Wegener's granulomatosis and have induced cures in 90% or more of these patients. We have now applied these approaches with remarkable success to other of the vasculitic syndromes including systemic vasculitis of the polyarteritis nodosa group, isolated CNS vasculitis, Takayasu's arteritis and recurrent panniculitis with vasculitis. Our categorization schemes and the therapeutic protocols designed by us are now widely accepted and are being utilized by medical centers throughout the world. We have established linkages between several of these diseases and various immune response genes as indicated by HLA-DR haplotype associations. These patient populations have been utilized to precisely delineate aberrations of activation and immunoregulation of lymphoid cell function in man and have served as excellent models for the concomitant study of the normal human immune system. In addition, the precise effects of various therapeutic regimens, particularly corticosteroids and cytotoxic agents, on human lymphoid cell activation and immunoregulation have been described. (Fauci, Cupps, Volkman, and Lane, LIR/NIAID).

Administrative, Organization and Other Changes

Towards the end of 1980, the Scientific Director, NIAID established the Laboratory of Immunoregulation as a new laboratory within the Intramural program. Dr. Anthony S. Fauci who was at the time serving as the Head, Clinical Physiology Section, LCI, NIAID as well as the Deputy Clinical Director, NIAID was made the Chief, Laboratory of Immunoregulation. The Clinical Physiology Section, LCI, NIAID was dissolved. Dr. Fauci will continue his duties as the Deputy Clinical Director, NIAID. The Laboratory of Immunoregulation will be devoted predominantly to the study of the mechanisms of activation and immunoregulation of human lymphocytes, particularly human B lymphocytes, in normal individuals and in a variety of disease states characterized by abnormalities of lymphocyte function.

The plan within the laboratory is to develop predominantly from the existing clinical associate and fellow pool a group of individuals who will be staying on for a number of years and who will ultimately assume senior positions in the laboratory. In this regard, Drs. David Volkman and Thomas Cupps are remaining on the LIR staff following the termination of their clinical associateship on June 30, 1981. In addition, Dr. H. Clifford Lane has been designated as an additional individual who will be put on the tract toward tenure. Dr. Lane will complete his clinical associateship in June 1982 and on July 1, 1982 will assume the role as a more senior person within the LIR. In order to expand the projects within the LIR, a number of new individuals have been added to the staff. These include Drs. Theresa Gerrard as a staff fellow, Dr. Marion Peters as a visiting fellow, Dr. Tsu Liping as a guest worker. In August or September of 1981, Dr. Atsuchi Muraguchi will join the laboratory as a visiting fellow. In addition, an allotment of two to three clinical associates a year will be assigned to the LIR as they enter the intramural program each year. The remainder of the clinical associates including Drs. Butler, Kerhl, Falkoff, and Harley will remain within the intramural program. Depending upon the availability of positions, individuals within the clinical associate pool will be staying on for an extra year or two to complete or implement ongoing projects.

With the opening of the ambulatory care research facility (ACRF), new space will be available within the intramural program. The LIR will assume the modules vacated by the LCI on the B wing of the 11th floor following the appropriate renovations. This additional space will allow facilities for the additional personnel who have been and will be added to the LIR staff.

Honors, Awards, and Scientific Recognition

Over the past year, members of the Laboratory of Immunoregulation, predominantly in the person of Dr. Fauci, Chief, LIR have received a number of awards and honors. This year Dr. Fauci served as President of the American Federation for Clinical Research. In addition Dr. Fauci was named to a number of committees of scientific note. He is serving on the Committee on Clinical Immunology and Immunopathology of the American Association of Immunologists. Dr. Fauci is also a representative of The American Association of Immunologists to the Examinations Committee of the American Board of Allergy and Immunology. In addition, Dr. Fauci is on the post-graduate education committee of the American Academy of Allergy and is a member of the prestigious Section on Physiology in Clinical Science of the American Physiology Society. It is of note that he is the only immunologist on this well known section on Physiology in Clinical Sciences which is organized and run by the American Physiology Society. In addition, he is a member of the subcommittee on the classification of vasculitis of the American Rheumatism Association and is on the ad hoc committee of the National Foundation for Infectious Diseases.

Dr. Fauci serves on a number of editorial boards of journals concerned with the areas of immunology, allergy, and infectious diseases. He is the Section Head of the Editorial Board of the Clinical Immunology Section of the Journal of Immunology. In addition, he is the Associate Editor in charge of Allergy and Immunology of the American Journal of Medicine. Dr. Fauci also serves on the editorial boards of The Journal of Clinical Investigation, The Journal of Immunopharmacology, The Journal of Clinical Immunology, the new series entitled Clinics in Immunology and Allergy, the journal Human Lymphocyte Differentiation, the new series Clinical Immunology Review and the new journal EOS. In addition, Dr. Fauci in collaboration with Dr. John I. Gallin of the LCI, NIAID, has created and is the co-editor of the newly established series Advances in Host Defense Mechanisms.

As part of the recognition for scientific accomplishments, clinical investigators may be asked to visit outside institutions and serve for periods of from two to three days as visiting professor within a given institution. In this regard Dr. Fauci had been asked and did serve as visiting professor at several major institutions during the year. Among these were his visiting professorships at the University of Pennsylvania School of Medicine; the University of Florida Medical Center in Gainsville, Florida; the University of Cincinatti; the Hahnemann Medical Center; and the Henry Ford Hospital.

In addition, Dr. Fauci was asked to give several major named lectureships during the year. He delivered the Wadsworth Memorial Lecture at Rush Presbyterian-St. Lukes Medical Center, Rush Medical College, Chicago, Illinois. He delivered the State of the Art Lectureship at the annual meeting of the Midwestern Section of the American Federation for Clinical Research in Chicago, Illinois. He delivered the Distinguished Visiting Lectureship at the Regional Meeting of the Royal College of Physicians and Surgeons of Canada. He delivered the Presidential Address at the National Meeting of the American Federation for Clinical Research in San Francisco, California in April, 1981.

Dr. David Volkman was asked to deliver an invited lecture at the minisymposium on B cell function sponsored by the American Association of Immunologists at their national meeting in Atlanta, Georgia. In addition, Dr. Fauci organized and ran the Scientific Workshop on Immunoregulation at the National Meeting of the American Rheumatism Association in Boston, Massachusetts.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00210-01 LIR

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Activation and Immunoregulation of Human Lymphocyte Function in Normal and Disease States.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. Anthony S. Fauci, M.D., Chief, LIR/NIAID

Other Investigators:
H.C. Lane, M.D., Clinical Associate, LIR/NIAID
D. Volkman, M.D., Ph.D., Medical Officer, LIR/NIAID
T. Cupps, M.D., Medical Officer, LIR/NIAID
R. Falkoff, M.D., Ph.D., Clinical Associate, LIR/NIAID
J. Harley, M.D., Ph.D., Clinical Associate, LIR/NIAID
H.C. Stevenson, M.D., Medical Officer, LIR/NIAID
J. Butler, M.D., Clinical Associate, LIR/NIAID
J. Kehrl, M.D. Clinical Associate, LIR/NIAID
M. Peters, M.D., Visiting Fellow, LIR/NIAID
T. Gerrard, Ph.D., Staff Fellow, LIR/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Immunoregulation, NIAID

SECTION

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland

TOTAL MANYEARS: 6	PROFESSIONAL: 4	OTHER: 2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A highly sensitive and reproducible system of antigen-induced, antigen-specific activation of human B cells was developed which led to a precise delineation of the dichotomy and overlap between antigen-specificity and polyclonality of in vitro B cell responses. The profile and kinetics of the circulating human antigen-specific B cell repertoire were described in this system and the heterogeneous mechanisms of modulation of B cell responses were precisely delineated. The system was T cell dependent and modulated by multiple interacting subsets of helper and suppressor T cells. B cells were also shown to be directly tolerized by high antigen concentrations. Monocytes were absolutely required for the system; they were suppressive in excess numbers; they presented antigen to the T cells in the T cell-monocyte-B cell interactions and this required strict HLA-DR compatibility between the T cell and monocyte. On the other hand, the B cell required direct interaction with the antigen and could not be triggered by antigen presented via the monocyte. The complex mechanisms and multiple sites of action of corticosteroids and cytotoxic agents on the activation and immunoregulation of human lymphocyte function were delineated, as were the abnormalities of immune function in various disease states.

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(Rev. 2-81)

Objectives:

The precise delineation of the mechanisms of activation and immunoregulation of human lymphocyte function, particularly B lymphocyte function, has been an area of intensive interest and study in human immunobiology. This interest stems from the fact that many of the human diseases which are mediated by immune mechanisms would require for their understanding a knowledge of the mechanisms whereby cells are activated and regulated. In this regard, this laboratory has played an active role over the past several years in describing some of the first in vitro systems whereby human B lymphocytes are activated and their immunoregulatory mechanisms delineated. In this regard, we had established polyclonal activation systems whereby cells were triggered nonspecifically and their specific antibody and total immunoglobulin production was measured. In addition, the multiple subsets of lymphocytes and monocytes which modulate these polyclonal systems were described. However, one of the main impediments to the full understanding of the complex mechanisms of B lymphocyte activation was the lack of a truly specific system whereby cells could be induced with specific antigen and antigen-specific responses could be adequately measured in vitro. The major difficulty has been an extraordinary overlap between polyclonality and specificity in the induction and expression of in vitro responses. One of the priority goals of the laboratory in this past year was to establish a truly antigen-induced, antigen-specific in vitro system for the study of human B lymphocyte responses. In addition, a major thrust of the laboratory was to precisely delineate the multiple complex mechanisms of regulating this antigen-specific system both from a cellular interaction as well as a humoral factor approach. This would require the precise identification and characterization by a number of techniques of the multiple subsets of cells involved in these immunoregulatory mechanisms. In addition, the mechanisms whereby B cells can be directly tolerized versus regulated by T cell interactions was a major goal and objective of the laboratory. Having established these systems to examine the immunoregulation of human B cell function, these approaches would then be applied to a number of disease states in which aberrations of lymphocyte function play a primary or secondary role. In addition to the above objectives, an effort was made to precisely delineate the genetic control of the immune response at the T cell, monocyte, and B cell-interaction levels similar to that which has been elegantly described in the animal models. Again, the reason for this approach has been evidence from this laboratory and other laboratories that several immune mediated diseases are associated with a particular genetic profile represented at the HLA-DR locus.

Finally, since many of the immune mediated diseases which are under study have been successfully studied with immunosuppressive agents, particularly corticosteroids and cytotoxic agents, a major effort was made to precisely delineate the mechanisms whereby these immunosuppressive agents modulate and influence the activation and immunoregulation of lymphoid cell responses. Particular emphasis is made in this laboratory on studying the above phenomena in the human model.

Methods Employed:

The predominant theme of this laboratory is the precise delineation of the complex mechanisms of immunoregulation in normal human immunologic reactivity and in disease states characterized by abnormalities of immunoregulation and/or the expression of immune reactivity.

In this regard, a major component of the methodology relates to the fractionation, identification and purification of immunoregulatory lymphoid cell subpopulations and the *in vitro* culturing of these cells to define their diverse functional capabilities. Subpopulation of cells are identified by surface markers and functional capabilities are assessed by a variety of *in vitro* assays. We have focused on the relationship between various *in vivo* and *in vitro* triggering signals and the subsequent expression of direct and immunoregulatory functional capabilities. This has been focused predominantly in the area of activation, proliferation and differentiation of human B lymphocytes to antibody producing and secreting cells. In this regard, we have been utilizing a unique model of human B cell function which was originally developed in this laboratory. It is a system of primary *in vitro* stimulation of bone marrow derived (B) lymphocytes by polyclonal activation as well as specific antigenic stimulation with subsequent measurement of single cell antibody production by a direct hemolysis-in-gel plaque forming cell (PFC) assay. We have further extended this system to now measure indirect (IgG) PFC. We also have developed a system for the measurement of Ig secreting cells of all classes by use of staphylococcal protein A (SPA) coated target erythrocytes and class specific developing antisera.

In addition, highly sensitive assays (enzyme linked immunosorbent-ELISA) have been developed and are being employed to measure the *in vitro* production of specific antibody following *in vivo* immunization of human subjects as well as primary *in vitro* antigenic stimulation.

In studies involved in the delineation of antigen specificity of human B cell responses, normal volunteers are immunized with various antigens such as keyhole limpet hemocyanin (KLH) and tetanus toxoid. Their lymphocytes are subsequently cultured as described above in order to determine the cellular interactions, genetic restrictions, role of presenting cells and role of specific and non-specific signals in the *in vitro* induction of antigen specific B cell responses. In addition, sera from immunized subjects are purified by affinity chromatography to obtain specific Ig of various classes in order that immune complexes may be made *in vitro*. These immune complexes are then employed in the *in vitro* culure systems to determine the role of specific IgM versus IgG antibody as well as immune complexes in various states of antigen or antibody excess in the induction and regulation of specific B cell responses.

The system of *in vitro* triggering of specific antibody production by human B cells is now being adapted to the hepatitis B system in order to study the immunoregulatory events operable in diseases such as certain of the vasculitides which are associated with aberrant immune responses to the hepatitis B antigen.

A system of in vivo priming with a carrier (KLH) and in vitro stimulation with a hapten-carrier conjugate (TNP-KLH) has been developed and adapted for use with human B cells.

Ricin, a substance highly toxic to cells, has been conjugated to a soluble antigen (tetanus toxoid) in order to directly remove tetanus toxoid reactive B cells by binding of the antibody combining site of the cell to the antigen bound to the toxic ricin.

Other assays employed to delineate the functional capabilities of lymphoid cells are the in vitro blastogenic responses to mitogenic and antigenic stimulation, elaboration of various soluble mediators, cell mediated cytotoxicity against various autologous, allogeneic and xenogeneic targets. Various cytotoxicity assays include spontaneous, mitogen dependent, and antibody dependent cellular cytotoxicity. A particular aim is to identify and characterize the abnormalities of lymphocyte or monocyte subpopulations involved in immunologically mediated diseases to determine the primary or secondary nature of these abnormalities and their relationship to the altered state of immune reactivity, as well as to create in vitro models of altered immunoregulation by selectively triggering or manipulating immunoregulatory subpopulations of cells.

Lymphocyte subpopulations are identified by unstable cell surface markers such as Fc receptors for various classes of Ig. In addition, heteroantisera against functional lymphoid cell subsets were employed. Recently, we have developed a panel of hybridoma monoclonal antibodies against functionally defined lymphocyte subsets as well as against lymphocyte subsets whose precise functional capabilities have not as yet been clearly defined. The use of hybridoma antibodies against lymphocyte subsets has proven invaluable as methodologic tools in the precise delineation of lymphocyte subsets in normal and aberrant immunologic reactivity.

Recently developed elutriation centrifugation techniques are being employed to isolate purified (greater than 95%) populations of human monocytes from peripheral blood, thus avoiding adherence techniques.

A major methodologic advance has been made in the laboratory by developing the first soluble antigen-specific T cell clones reactive with the antigen KLH. We have employed these clones to dissect the functional properties of pure populations of human T cells with regard to the immunoregulation of B cell responses.

Bone marrow aspirates are performed in normal subjects and patients with various diseases prior to, during and following therapy. Purified populations of lymphoid cells are obtained from these bone marrow cell suspensions by various separation techniques in order to delineate the immunocompetence of bone marrow lymphoid cells in normal man, as well as alterations of compartmentalization and function of these cells in untreated and treated disease states.

Corticosteroids are administered to normal volunteers and patients requiring this drug and selective effects of these agents on the compartmentalization and functional capabilities of monocytes and lymphocyte subpopulations are determined.

In addition, the differential effects of in vitro corticosteroids and in vitro irradiation on various lymphoid cell functional capabilities such as triggering, proliferation, differentiation, antibody production and secretion are being studied. In patients receiving cytotoxic agents (cyclophosphamide, azathioprine, etc.) for the therapy of immunologically mediated diseases, studies are performed on lymphoid cells in the same manner as that mentioned above for corticosteroid administration to normal volunteers and patients.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

A) Antigen-induced, antigen-specific activation of human B cells.

1) Development of in vitro antigen-induced, antigen-specific B cell responses.

The mechanisms of activation and immunoregulation of human B cell function have been the subject of intense interest and investigation in immunobiology over the past several years. Several years ago this laboratory developed the first polyclonally induced in vitro system for the measurement of antibody production by human B cells at the single cell level using a hemolysis-in-gel plaque forming assay. A number of important observations were made regarding the activation and immunoregulation of human B cells by this system. However, the study of human B cell activation and immunoregulation has been hampered by the lack of a reproducible, artifact-free, truly antigen-specific system to induce specific antibody in human B cell in vitro. Over the past year, this laboratory has successfully developed the first truly antigen-induced, antigen-specific system to measure human B cell responses against soluble antigens. The antigens used were keyhole limpet hemocyanin (KLH) and tetanus toxoid (TT). The success of the system was based on the realization that antigen reactive B cells are present in the circulation for only a limited time following in vivo immunization. Previous attempts to induce a primary in vitro response with specific antigen had failed for a number of reasons including, most importantly, the lack of available antigen specific cells in the circulating B cell repertoire. Therefore, a system was set up whereby normal volunteers and certain patient groups were immunized in vivo with the soluble antigen KLH and/or TT. The subjects were boosted two weeks following the primary immunization and two weeks following the booster immunization their cells were examined for the ability to be triggered in vitro with the specific antigen in question. A number of interesting and important observations were made. Previous attempts at stimulating cultures with specific antigen had used concentrations of antigen which rendered maximal blastogenic responses, most likely at the level of the T cell. Thus, in attempts to trigger B cell responses, similar concentrations of antigen were used. By a series of pilot experiments, it was learned that he optimal concentration of antigen that was needed to stimulate B cells was orders of magnitude less than the optimal concentration needed to trigger T cell blastogenesis. In fact, the most important observation was that the concentrations of antigen that actually triggered T cell blastogenesis were directly suppressive to the B cells in question. Therefore, although a number of other studies from this and other laboratories were able to demonstrate that mitogens such as pokeweed mitogen (PWM) were able to trigger the production of specific antibody along with the necessarily concomitant polyclonal Ig response, the introduction of specific antigen in the concentrations commonly used into the culture did not trigger B cells to secrete specific antibody. The fact is that such concentrations were actually suppressive as opposed to being incapable of triggering the antigen-specific B cells in question. Another observation of extreme importance in the successful development of this system was the fact was that associated with the antigen-specific induction of antibody responses *in vitro* was a necessarily concomitant polyclonal Ig response. This interfered with the ability to precisely examine antigen-specific responses alone. After a number of preliminary experiments, it was learned that the actual culture conditions which are commonly used for such systems, i.e. round bottomed culture tubes, were condusive to the development of a polyclonal response simultaneously with the antigen specific response. By altering the culture conditions and using flat bottomed wells as opposed to round bottomed tubes, we were able to dissociate this otherwise invariable overlap between polyclonal Ig production and specific antibody production in response to the antigens in question. Therefore, under well defined culture conditions, we were able to demonstrate the first truly antigen-induced, antigen-specific triggering of circulating human peripheral blood B lymphocytes. This system has been and will now be used to dissect out the precise mechanisms of activation and immunoregulation of human B cell function in normal individuals and in a number of disease states characterized by abnormalities of immune function.

2) State of activation and memory of the circulating B cell repertoire.

Once having established the successful induction of antigen-specific antibody responses *in vitro* by human B cells as described above, the *in vivo* and *in vitro* kinetics of the system were precisely delineated. It was found that circulating in the peripheral blood in a time dependent manner following *in vivo* immunization were multiple subsets of B cells at various states of activation and capable of being triggered to secrete specific antibody by a number of diverse stimuli. A few days following booster immunization *in vivo*, there were present in the circulation substantial numbers of B cell blast-like lymphocytes which were spontaneously secreting Ig, predominantly of the IgG class. These cells secreted antibody without the requirement for T cell help and were shut off if other mitogenic stimuli or antigen were introduced into the system. These cells soon disappeared from the circulation and were replaced by cells which were able to be triggered either by specific antigen or PWM. The persistence of antigen reactive B cells in the peripheral blood following immunization depended upon the duration of time following the *in vivo* booster. In most individuals, maximal ability to trigger B cells was observed approximately two weeks following the *in vivo* boost. The duration of the circulation of this B cell repertoire which could be triggered by antigen varied considerably from individual to individual. In addition, in most individuals a marked shift in the expression of isotype was observed. Within weeks following immunization, the predominantly secreted isotype was IgM specific antibody. However, when these same individuals were assayed approximately one year following the *in vivo* immunization, a dramatic switch to the IgG isotype was observed. Studies are currently in progress to determine if the expression of isotypes is controlled at the circulating B cell repertoire level or by isotype specific helper T cells.

3) T cell and monocyte dependence.

Similar to the previously studied PWM-induced secretion of total Ig, the antigen-induced, antigen-specific triggering of human B cells was both T cell and monocyte dependent. Of particular note was the difference of degree of dependence of the PWM and antigen-specific system for T cells and especially monocytes. This led us to embark upon a new series of experiments to more precisely fractionate and/or deplete various lymphocyte subsets as well as monocytes from the cell suspensions to be tested. It was learned that PWM stimulation was much more dependent on the presence of monocytes and that virtually all monocytes needed to be depleted from the mononuclear cell suspensions in order to dampen the antigen-induced responses. In addition, a number of studies were performed to precisely delineate the lymphocyte subsets required for the activation and regulation of this antigen specific B cell response.

4) Development of hapten-carrier systems for the induction of specific B cell responses.

Utilizing the systems whereby normal volunteers and patients were stimulated _in vivo_ with carriers such as KLH and TT, a novel system utilizing hapten-carrier triggering _in vitro_ was employed. Individuals who were immune to the carrier KLH and/or TT were assayed for the ability of their cells to be triggered _in vitro_ with a hapten conjugated carrier. Specifically, TNP was conjugated to the carrier and cells were stimulated _in vitro_. It was found that individuals who were primed to the appropriate carrier were able to express an antigen-specific response to the hapten alone. Of particular note is the fact that concentrations of the carrier such as KLH which resulted in suppression of the KLH specific response, i.e. high concentrations of carrier, did not suppress the ability of the TNP-specific B cells to mount an anti-TNP response to stimulation with TNP coupled to the KLH carrier. This proved that the suppression of KLH responses soon at the high concentrations of KLH was a true tolerization of the KLH specific B cells and was not a blocking at the T cell level since the KLH carrier was able to supply ample T cell carrier help to the TNP specific B cells.

5) Selective depletion of antigen-reactive B cells by the cytotoxic agent ricin.

It would be of particular clinical relevance to be able to eliminate specifically and exclusively antigen reactive B cells. This would have obvious implications in the elimination of hyperreactive B cells in certain hypersensitivity and autoimmune states. One of the methods of specifically eliminating such B cell subsets is to poison the cells by a technique which would damage only the antigen reactive B cells in question. In this regard the powerful cytotoxic substance ricin was conjugated to a given antigen, in this case either KLH or TT. The ricin conjugated carrier was then introduced into a suspension of cells containing the antigen reactive B cells. The antigen bound to the B cells in question and thus brought the ricin in close enough proximity to damage only the B cell which had bound the antigen. In this respect, only the antigen specific B cells were eliminated by the cytotoxic ricin. Further studies are in progress now to determine if other antigen specific B cells could be selectively eliminated by such directed cytotoxic techniques.

B) Immunoregulation of antigen-specific B cell responses.

1) Studies of B cell tolerance.

Utilizing the newly developed antigen-induced, antigen-specific in vitro activation of human B cells as described above, a number of studies directed at the delineation of the antigen-induced suppression of B cell function were undertaken and were extremely productive. It was found that at high antigen concentrations introduced into culture, the antigen in question reacted with the antigen specific T cells resulting in a substantial T cell blastogenic response. In addition to this blastogenic response, nonspecific T cell factors were elaborated which triggered multiple clones of B cells to secrete polyclonal Ig. In other words, high concentrations of antigen such as KLH in a KLH-immune individual resulted in an amplification of the response in vitro which led to the triggering of multiple clones of B cells. However, this same antigen concentration which triggered polyclonal Ig response resulted in no immunoglobulin production of the antibody specificiity related to the antigen in question. A number of studies definitively proved that the unresponsiveness to the specific antigen introduced in culture was due to a direct tolerization of the B cells in question and was not due to the induction of antigen-specific or even nonspecific suppressor T cells. This was demonstrated by preexposing various subsets of T cells as well as B cells with the antigen in question as well as by eliminating suppressor subsets of cells by the use of hybridoma antibodies and complement to specifically remove various functional subsets of T cells. The exact kinetics and duration of tolerance induction in this system are now under intensive study.

2) Relative irradiation sensitivity of immunoregulatory lymphocyte subsets in antigen-specific versus polyclonal systems.

It is of extreme importance to determine the relative sensitivity and lack thereof of various subsets of lymphocytes, namely B cells as well as immunoregulatory T cells, to immunomodulatory manipulations. The two most important of these are immunosuppressive agents such as corticosteroids and cytotoxic agents as well as irradiation. In the series of studies to be described in this summary, cells were irradiated with various doses of irradiation and the relative sensitivity of immunoregulatory lymphocyte subsets wwa delineated. We had previously published that suppressor T cells were relatively sensitive to irradiation whereas helper T cells were markedly resistant to irradiation. These studies were done in the mitogen-induced Ig production systems. This work was corroborated by a number of other laboratories. However, in the above described antigen-specific system, it was found that the relative irradiation sensitivity profile was somewhat different. In brief, it was found that although the T cells required for the T cell dependent polyclonal induction of Ig responses were quite resistant to irradiation, the antigen-specific T cells required for the specific induction of T cell dependent specific antibody were, as a matter of fact, relatively sensitive to irradiation and could be eliminated by doses of irradiation over 500 rads. This dose had absolutely no effect on the T cells required for polyclonal activation.

3) Role of allogeneic effects in antigen specific B cell systems.

Any study of in vitro human lymphocyte function requires precise knowledge of the role of allogeneic effects on the responses being measured. This is necessary since many, if not most, of the studies of patient populations with abnormal immune functions are predicated on the ability to co-culture the patients cells with various fractions of normal allogeneic cells. In this regard, it was found in our previously published mitogen-induced Ig production systems that allogeneic effects enhanced the system and did not in any manner or form interfere with the production of immunoglobulin. By a series of experiments aimed at this question in the antigen-specific system, it was found that allogeneic effect factors were able to replace T cells under certain circumstances. However, it was found that when T cells from one individual were cultured with B cells and monocytes from an allogeneic individual, allogeneic suppression of the B cell responses resulted. This is of critical importance to appreciate if one is to delineate the genetic restriction related to such B cell responses. In other words, if genetic studies are to be performed and the system is suppressed by allogeneic effects, then one would not be able to determine if the lack of response was due to an allogeneic suppression or to a true genetic restriction.

4) Role of T cell subsets in the regulation of polyclonal versus antigen specific systems.

Precise delineation of the immunoregulatory events associated with the regulation of human B cell responses requires a knowledge of cellular interactions at the T cell subset level. We and others have previously delineated the requirement for helper and suppressor T cells as determined by cell surface receptors as well as cell surface antigens as defined by monoclonal antibodies. The delicate balance between helper and suppressor influences were amply demonstrated in our previously described systems. Using the newly developed antigen-specific B cell system described in the above sections, the precise delineation of the T cell subsets involved in the antigen-specific versus polyclonal induction of B cell responses was accomplished. It was learned that significant differences exist in the relative sensitivity to modulation by immunoregulatory T cell subsets comparing the antigen-specific and polyclonal system. Very small subsets of T cells were responsible for the immunoregulation of the antigen-specific system while broad subpopulations of cells described by the OKT 4 and OKT 8 markers as well as by other markers related to monoclonal antibodies modulated the polyclonal B cell responses.

5) Role of the macrophage in the immunoregulation of B cell responses.

Again, using this newly developed antigen-specific B cell system we were able to carefully examine the role of the macrophage in the activation and immunoregulation of human B cell responses. This is of particular importance given the well known role of the macrophage in modulating immune responses in normal and disease states. We had proven, along with other investigators, in the polyclonally induced system some years ago that macrophages were both required for the system and could induce suppression. In this regard, we demonstrated that a substantial degree of macrophage related suppression was seen in certain diseases such as sarcoidosis and active tuberculosis. However, as with most polyclonal systems, this particular arena was clouded by the fact that we were dealing with a total Ig response and not with a specific antigen. Therefore, intensive studies were carried out to delineate the role of the macrophage in this antigen-specific system. It was found that indeed the macrophage was required for the antigen-induced, antigen-specific triggering of B lymphocytes. In addition, excess macrophages resulted in the same type of suppression that was observed with the polyclonal system. Of particular importance was the question of whether the macrophage functioned as a presenting cell used to present antigen in its native or modified form to the T and/or B cell as has been demonstrated in the animal model. A number of interesting observations were made. The macrophage indeed was required for T cell blastogenesis in response to the antigen in question in this antigen-specific B cell system. In other words, the system was T cell dependent and the T cell required the macrophage in order to respond to the antigen. Therefore, indirectly the B response itself was dependent upon macrophages. Of particular importance was the fact that the macrophage-T cell interaction was strictly genetically controlled. Identity of at least one of the two HLA-DR haplotypes was required between the monocyte and the T cell in order for the antigen to induce substantial T cell blastogenic responses. As will be discussed in another section, this absolute DR restriction was proven at the clonal level in a series of studies employing soluble antigen-specific T cell clones. Of particular interest was the fact that one could pulse monocytes with pokeweed mitogen in the induction of the pokeweed mitogen triggered T cell dependent B cell responses. However, one could not pulse monocytes with antigen in the induction of antigen-specific B cell responses. This was a marked contrast to the observation that one could successfully pulse monocytes to induce T cell responses. In other words, the T cell which expresses a relatively weak antigen receptor required the cooperation of monocytes to respond to the antigen in question. On the other hand, the B cell which expressed a high affinity Ig receptor binds to the antigen in question and only in the presence of the monocyte-T cell interaction does the B cell then become triggered to secrete antibody. Thus, the monocyte is required for the T cell recognition of antigen but the B cell itself must see the antigen exclusive of any other cooperating cell types. These studies will undoubtedly delineate the complex interactions required for the successful triggering and immunoregulation of human B cell responses. Finally, the role of soluble monocyte factors, ie. interleukin 1 (IL-1), were precisely described.

6) Immunoregulation by antigen specific hybridoma antibodies.

A series of experiments were successfully completed establishing a mouse-human heterohybridoma by fusing a KLH reactive human circulating B cell with a HAT sensitive mouse myeloma cell. The heterohybridoma made large quantities of human monoclonal anti-KLH antibody. This antibody was used to study the effect on the modulation of B cell triggering and its immunoregulation. In an interesting and exciting series of experiments, it was found that the monoclonal anti-KLH which was certainly of a well defined idiotype was actually mitogenic for the KLH specific B and/or T cells. The implications for this observation related to the anti-idiotype regulation of B cell responses is obvious. Studies are currently being pursued to determine the mechanisms whereby this monoclonal anti-KLH antibody can be mitogenic in a system containing KLH immune B and/or T cells.

C) Pharmacologic Modulation of Human Immune Responses.

1) The role of in vivo and in vitro corticosteroids on human lymphocyte functions.

Corticosteroids are widely used in clinical medicine to modulate inflammatory and/or immune response in patients with diseases characterized by hyperreactivity of immune function. In this regard, this laboratory has been involved in the study of the effects of in vivo and in vitro corticosteroids for the past several years. In previous studies, the selective effects of in vivo corticosteroids on immunoregulatory T cell subsets was clearly delineated. Over the past year, these studies have been extended and the diverse effects of in vitro corticosteroids on human B cell function and its immunoregulation were precisely described. It was found that in vitro corticosteroids resulted in an increase in the spontaneous secretion of Ig by resting B cells. This was not due to a selective killing or removal of a lymphocyte subset but was probably due to a modulation of the ability of the cells to express and secrete immunoglobulin. In addition, the same in vitro corticosteroid had no significant effect on pokeweed mitogen stimulated Ig secretion by B cells. On the other hand, treating of normal volunteers or patients with corticosteroids in vivo resulted in a marked suppresion of the B cell responses. This was due at least in part to a T suppressor cell. The mechanism involved in this suppression was likely a shift in the proportion of immunoregulatory T cell subsets resulting in a greater relative number of suppressor T cells as compared to helper T cells. The mechanism for the shift in proportion had been shown by us some time ago to be a selective depletion from the circulation of helper T cells. In addition to the effect on B cells of in vitro corticosteroids, an interesting and exciting result was found in the study of the effect of in vitro corticosteroids on T cell markers as determined by monoclonal antibodies. It was found that certain markers of activation delineated by the 3A1 and 4F2 monoclonal antibodies developed in this laboratory were expressed to a greater degree in cultures which had been exposed to corticosteroids. In other words, a modulation of the activation of T cells was accomplished by the presence of corticosteroids in culture. Another interesting and exciting observation was the fact that following treatment in vivo with corticosteroids, there appeared in the circulation together with the relative T lymphocytopenia the presence of T cells which expressed markers which are seen only on thymocytes and immature T cells. This marker, the OKT-6 marker, is generally found on only a small percentage of circulating T cells. During and following corticosteroid treatment this marker is seen in a significantly higher degree suggesting that immature T cells have entered the circulation concomitant with the treatment with corticosteroids.

2) The role of cytotoxic agents in the activation and immunoregulation of human B cells.

This laboratory has been studying the role of cytotoxic agents in the modulation of human immune function for several years. The major reason for this interest is the fact that several of our patient populations are being treated with cytotoxic agents, particularly cyclophosphamide and to a lesser degree azathioprine for immune mediated diseases. In a series of experiments conducted several years ago, it was found that cyclophosphamide had a relatively greater affect on B cells as compared to T cells. Within the T cell population, it was found that suppressor cells were relatively more sensitive to cyclophosphamide then were helper T cells. In the series of experiments conducted over the past year, an interesting and exciting group of observations were made. It was found that cyclophosphamide therapy in vivo had a profound suppression of the ability of B cells to secrete immunoglobulin. This affect was highly selective at the B cell level with relatively little affect on helper or suppressor T cells in the concentrations of cyclophosphamide used in our patient populations. Of particular note was the fact that equivalent dose regimens of azathioprine therapy had no similar suppressor affect on the B cell function of our patient population. This later observation is of particular importance given the fact that we have found cyclophosphamide to be significantly more effective in the control of B cell mediated disease than azathioprine. This selective suppression of B cell function by cyclophosphamide may well explain this consistent clinical observation.

D) Generation and Expression of Cytotoxic Cell Activity In Vitro.

1) MLR-induced generation of NK, ADCC, CTL.

Multiple categories of cytotoxic cells most likely play a significant role in various immunologic phenomena in normal and disease states. The three most studied of these are the cytotoxic T lymphocyte (CTL), the natural killer cell (NK), and the antibody dependent cellular cytotoxicity system (ADCC). Over the past several years, this laboratory has been involved in delineating the cells types involved and the mechanisms of killing in the NK, ADCC, and CTL systems. Of particular interest were the mechanisms of the generation and expression of cytotoxic cell activity following the mixed lymphocyte reaction (MLR). Also of interest was the description of the phenotypic expression of the various components of the cytotoxic reactions in order to identify them in their generation and expression. MLR-activated T cells were analyzed according to the expression of various cell surface markers by the CTL generated in the MLR. CTL were found exclusively in a population of MLR-activated T cells that lacked detectable Fc receptors for IgG but that expressed a surface antigen recognized by the 4F2 monoclonal antibody. In contrast, CTL were found in both the Ia-positive and Ia-negative cells after MLR activation. Thus, the specific CTL generated in the allogeneic MLR can be identified and isolated by virtue of the expression of a particular cell surface marker. In addition, the phenotypes of the various MLR-activated T cell populations which mediate cytotoxicity in different effector cell assays such as NK, CTL, and ADCC were studied. CTL mediating specific lysis of target cells bearing the stimulating alloantigens could clearly be distinguished from cells mediating ADCC in that CTL were Fc receptor (IgG) negative while cells mediating ADCC were Fc receptor (IgG) positive. In contrast, MLR-generated NK cells were distributed in both the Fc receptor positive and Fc receptor negative cell fractions.

The presence or absence of Ia antigens did not correlate with any of the cytolytic activity studied. In addition, Fc receptor negative and not Fc receptor positive cells responded in the secondary MLR. Thus, although Fc receptor positive cells generated in the allogeneic MLR played little, if any, direct role in specific cytotoxicity, they nonetheless are generated in large numbers by the MLR. However, their ability following MLR generation to mediate NK and ADCC, both of which may contribute to in vitro and in vivo lysis of allogeneic cells, might explain their appearance following allogeneic stimulation.

2) Mechanisms of cytotoxic cell function.

We have demonstrated the regulation and modulation of human natural killer (NK) cell activity by cyclic nucleotides and their intracellular inducers. The direct addition of the cyclic adenosine monophosphate (cAMP)-inducers dibutyryl cAMP (DB-cAMP), isoproterenol, aminophylline or histamine to peripheral blood mononuclear cell-K562 cell mixtures at the initiation of a 4 hr. 51-chromium-release microcytotoxicity assay significantly suppressed NK function in a dose-dependent manner. Cyclic guanosine monophosphate (cGMP) analogs or compounds known to increase the cGMP content of lymphocytes produced a small but signfiicant enhancment of cytotoxic activity. The continuous presence of these agents during the assay was not required for the observed effect. The cAMP and cGMP-induced alterations of killing occurred relatively early in the lytic process and the observed effects were apparent throughout the assay. Pre-incubation of effector cells with cAMP or cGMP-inducers prior to the NK assay produced transient, reversible modulation of lysis; the cyclic nucleotide-induced alteration in cytotoxicity lasted only 2 hrs. before returning to baseline pre-treatment values. cAMP and cGMP-induced changes in NK activity were reversible by specific antagonists. Blockade of the surface neurohumoral receptors for isoproterenol and carbachol by propranolol and atropine respectively abrogated the attenuation of NK function induced by these autonomic agonists. Experiments in which highly purified fractions of NK-enriched large granular lymphocytes (LGL) were employed indicated that cAMP- and cGMP- inducing agents affected activity of cytotoxic lymphoytes directly rather than through effects on accessory non-lytic cells. The cyclic nucleotide-induced alterations in NK activity were not due to changes in effector cell viability, target cell binding, or the susceptibility of target cells to lysis. These studies demonstrate the important roles of cyclic nucleotides in modulating and regulating NK function and may provide the basis for the eventual elucidation and manipulation of cytotoxic activity in normal and disease states.

E) The Role of Human Monocytes in the Production of the Alpha and Gamma Interferons.

Elutriation is a precise and efficient method for separating monocytes (MO) from lymphocytes (LY) on the basis of size. Elutriator-purified MO and LY were assayed for their ability to produce alpha and gamma interferons (IFN) in response to a variety of stimuli. All of the alpha IFN produced by unfractionated mononuclear cells in response to live Sendai virus was found to be produced by MO and not LY. MO produced 10,000 alpha IFN units per 10^7 cells, whereas LY produced less than 30 units per 10^7 cells. When MO were added back to LY prior to Sendai virus exposure, all of the alpha IFN produced could be accounted for by the MO in the cell preparation. Alpha IFN production by MO begins within 4 hours of Sendai virus exposure and is maximal at 24 hours post-viral infection. No alpha IFN was produced when MO were exposed to pokeweed mitogen (PWM), Con A, or PHA. In contrast, gamma IFN is produced exclusively by LY in response to PWM, Con A, or PHA. Since certain of these lectins (particularly PWM) bind preferentially to monocytes at low concentrations, the production of gamma IFN by LY can be a MO-dependent process, depending on the concentration of stimulating lectin employed. Accordingly, lectin-pulsed MO can be used to stimulate gamma IFN production by unpulsed LY. However, the MO-dependence of lectin-induced gamma IFN production by LY can be negated by employing higher concentrations of lectins. The results indicate that under the conditions employed in the present study, human MO are the sole producers of alpha IFN in response to Sendai virus and are potential modulators of the production of gamma IFN by LY in response to certain lectins.

F) Activation of Human B Cells in Parasitic Disease.

Disseminated cutaneous leishmaniasis (DCL) is a rare form of chronic leishmania infection in which there is a large number of organisms in the involved skin. Other protozan diseases such as malaria and trypanosomiasis are characterized by polyclonal hypergammaglobulinemia and variable levels of autoantibodies. The mechanisms of this polyclonal hypergammaglobulinemia are unclear. The presence of circulating B cells that spontaneously secrete immunoglobulin (Ig) is felt to be an indication of *in vivo* polyclonal activation. Thus, 4 patients with biopsy and culture proven DCL of greater than 5 years duration were studied for the presence of spontaneous Ig producing circulating lymphocytes by a reverse hemolytic plaque forming cell (PFC) assay using staphylococcal protein A conjugated to sheep erythrocyte targets and developing antiserum. Three of the 4 patients had markedly elevated numbers of spontaneous PFC compared to 17 normal individuals. One patient had normal numbers of spontaneous PFC. The presence of increased numbers of spontaneous PFC correlated with elevated serum levels of Ig, with parasite burden and with severity of disease. The patient with normal numbers of PFC had normal serum Ig levels and the least extent of skin involvement. Since certain parasitic antigens have been shown to be polyclonal B cell activators, it is likely that the *in vivo* polyclonal triggering of B cells demonstrated in the present study results directly or indirectly from the parasite itself and explains the polyclonal hypergammaglobulinemia in this disease.

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PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Study of Human Lymphocyte Subsets Employing Cloning and Hybridoma Technology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Anthony S. Fauci, M.D., Chief, LIR/NIAID

Other Investigators:

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H. Clifford Lane, M.D., Clinical Associate, LIR/NIAID
Joseph Butler, M.D., Clinical Associate, LIR/NIAID
Benjamin Sredni, Ph.D., Visiting Scientist, LI/NIAID
Ronald H. Schwartz, M.D., Ph.D., Senior Investigator, LI/NIAID
Barton F. Haynes, M.D., Dept. of Medicine, Duke University Medical Center

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Immunoregulation

SECTION

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse hybridoma monoclonal antibodies directed against surface antigens on subsets of human mononuclear cells were developed. The functional capabilities of these cell subsets were extensively studied and carefully delineated. The monoclonal antibodies defined: a) cells with inducer T cell function for help and suppression; b) states of activation; c) expression of antigens following MLR activation which could be used to dissect out CTL, NK, and ADCC populations; d) clonal expansions of cells in certain lymphoproliferative states such as the Sezary syndrome. In addition, a mouse-human heterohybridoma secreting pure monoclonal human anti-KLH antibody was established. The antibody is mitogenic for lymphocytes from KLH immune individuals but not KLH non-immune individuals suggesting a relevant idiotype-anti-idiotype interaction with the KLH immune cells. The first human soluble antigen-specific T cell clones were established and were shown to be true clones, antigen-specific, requiring HLA-DR compatible presenting cells. They were characterized phenotypically and were shown to express the OKT8+, OKT4- suppressor/cytotoxic phenotype which correlated with their in vitro suppressor cell function in our newly developed antigen-induced, antigen-specific system of human B cell activation.

Objectives:

The Laboratory of Immunoregulation has been intensively involved in the study of the mechanisms of activation and immunoregulation of human lymphocyte function. Over the past several years, two of the most important innovations in technology employed in the study of lymphoid cells has been the development of lymphocyte clones as well as the development of monoclonal hybridoma antibodies reactive with various functionally defined subsets of cells. Over the past year, we have been involved in developing a number of hybridoma monoclonal antibodies which define functional subsets of human lymphocytes. The objective of this particular study was to enable us to dissect out in a more precise manner the lymphocyte subsets involved in the immunoregulation of T and/or B cell function. In that regard, a number of hybridoma antibodies were developed against human lymphocyte antigens.

In order to study in a more exact manner the lymphocyte subsets involved in the immunoregulation of B cell function, attempts were successfully made to develop clones of T lymphocytes such that immunoregulation could be studied at the clonal level. Using the combination of clones of cells and antibodies which can recognize precise subsets of functional lymphoid cells, a major effort was placed in establishing an even more precise delineation of the mechanisms of activation and immunoregulation of human B cell function.

Methods Employed:

1) Employing the elegant experimental design of Milstein and Kohler for the production of somatic cell hybrids, we set out to develop monoclonal antibodies against human lymphocyte subsets. In this regard, mice were immunized with the T cell line HSB and a number of hybrids were formed by fusing the spleen cells from the immunized mouse with the HAT sensitive P3X63/Ag8 mouse myeloma. After appropriate screening procedures, a number of interesting and potentially important monoclonal hybridoma antibodies with specificity against human lymphocyte subsets were established.

2) These monoclonal antibodies after appropriate screening were catergoized as to the subsets of cells with which they reacted. Lymphocyte subsets were identified by direct fluoresceinating of the monoclonal antibodies followed by binding to the lymphocyte subsets in question and either direct visualization under the fluorescent microscope or by the use of the fluorescence activated cell sorter (FACS).

3) Functional lymphocyte subsets were removed from unfractionated mononuclear suspensions either by sorting on the FACS or by making use of the complement fixing capability of certain of these monoclonal antibodies. In the situations where the monoclonal antibodies fixed complement and were cytotoxic, the respective lymphocyte subsets to which the monoclonal antibodies bound were removed by cytolysis.

4) The lymphoid cells in the peripheral blood and lymphoid organs (when available) of certain patients with various immunologic mediated diseases were examined with regard to the relative proportions of these lymphocyte subsets as defined by the monoclonal antibodies which had been developed.

5) For the establishment of antigen specific human T cell clones reacting with soluble antigens the following methodology was employed. Normal volunteers were immunized with the soluble antigen keyhole limpet hemocyanin (KLH). After booster immunization the peripheral blood lymphocytes from the individuals were subjected to the standard cloning technology in soft agar by employing the techniques of Rozenszajn, Srendi, and Schwartz who had succeeded in developing T cell clones in the animal model. The clonality of the cells was proven by the appropriate expansions and subcloning.

6) Following the development of soluble antigen specific T cell clones, coculture experiments were performed in the autologous, DR compatible, and allogeneic systems to determine the genetic restrictions of the clones in question.

7) The clones were tested for their functional capability by culturing in an antigen specific system with autologous semi-allogenic and allogeneic B cells and monocytes from individuals immunized and not immunized with the KLH antigen.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

A) Monoclonal hybridoma antibodies directed against human mononuclear cell subsets.

1) Development of hybridoma monoclonal antibodies.

In order to more precisely delineate the immunoregulatory lymphocyte subsets involved in the regulation of human lymphocyte (particularly B cell) function, we developed a panel of mouse hybridoma monoclonal antibodies against human mononuclear cell subsets. This proved to be extremely rewarding from several standpoints.

We described in detail adaptations and modifications of the original Milstein and Kohler methodology for hybrids. In these studies we described revised screening strategies which allowed us to be successful in establishing a number of hybridomas. We went on the describe several (five) in some detail.

The hybridoma which we have studied in most detail up until now is the 3A1 antibody. It reacts with approximately 85% of peripheral blood T cells and is an IgG_1 k. We described the blastogenesis profiles in which 3A1 positive T cells respond suboptimally to PHA and only minimally to Con A. Clearly most of the classic "T_G" cells are 3A1 negative; however, it is also clear that a percent of T_G cells (E rosette positive, Fc_G receptor positive) are indeed 3A1 positive. This establishes along with other data from this laboratory that the T_G cell is a heterogeneous population of cells which includes T cells and most likely monocytoid cells as suggested by data from other laboratories. We further went on to describe that the 3A1 positive T cells were the helper cells in mitogen-induced B cell function. In addition, the

Con A generated suppressor cells were contained in the 3A1 positive population, whereas the "spontaneous" suppressor cells were contained in the 3A1 negative population which was predominantly T_G. These findings have been of major importance in clarifying the controversy regarding the difference between inducible and spontaneously occurring suppressor cells. In addition, we demonstrated that the 3A1 positive cells did not mediate ADCC while the 3A1 negative cells did.

2) Use of hybridoma antibodies reactive with human lymphocyte subsets to define clonal expansions in disease states.

Of particular excitement was the finding that the Sezary T cells is 3A1 negative while still maintaining its functionally mature helper capabilities. This suggests that the Sezary cell lost its 3A1 antigen during malignant transformation and so exemplifies a clearcut dichotomy between a surface marker which represents a population of normal helper cells and the expression of this helper capability by malignant cells which do not express this antigen. Such findings have potentially extremely important implications in our understanding of the molecular events at the cell surface level which are associated with malignant transformation of lymphoid cells.

In this regard, we were fortunate enough to have come upon a patient with what appeared to be a "malignant proliferation" of T_G cells. Given the disagreements in the literature regarding whether what investigators have been calling T_G cells are indeed T cells, we studied this patient in detail. In one complete study, we have demonstrated that this patient had a proliferation of normal appearing and functioning cells which were low affinity E rosette positive and Fc_G receptor positive, hence a classic T_G cell. However, most importantly, these cells were 3A1 positive and so clearly were T cells. Of particular note is the finding that this patient's expanded T_G population elaborates immune interferon spontaneously (without recognizable exogenous stimuli) in vitro. This phenomenon has not previously been described for interferon producing cells.

3) Use of hybridoma antibodies to delineate states of activation of human lymphocyte subsets.

Another antibody which we have described is the 4F2. It is an antibody which binds to monocytes and not to resting peripheral blood lymphocytes or even lymphoid organ lymphocytes. However, upon activation of lymphocytes, the 4F2 antigen is expressed. The antigen is not Fc receptor, not Ia and not HLA. Thus, there is an antigen which is normally found on resting monocytes, but only found on activated lymphocytes. This finding has important implications in our understanding of the relationship between monocyte and lymphocyte lineage and also may serve as a unique marker of lymphocyte activation exclusive of Ia and Fc receptors.

We have also described the 5E9 antibody. It appears to be leukemic specific in that it binds to all acute lymphoblastic leukemia (ALL) T cell lines tested, it binds to certain EB virus transformed B cell lines, to Burkitt's lymphoma cells, acute myeloblastic leukemia (AML) cells, 30% of null cell ALLs and to some T cell ALLs. Yet, it does not bind to normal thymus, spleen, peripheral blood cells or to normal continous T cell (CTC) lines. Thus, it is leukemic specific, but not leukemic type-specific. In addition, it is expressed on activated lymphocytes and so can be employed as another parameter of activation in normal and disease states.

Other antibodies which we are investigating from our panel is 3F10 which is an anti-HLA antibody similar to Milstein's W632. 3A6 is similar to 3F10 except that 3A6 blocks the binding of T cell growth factor (TCGF) to T cells whereas 3F10 does not. 5A12 antibody is essentially the same as 3A1 except that 3A1 is an IgG$_1$, k, while 5A12 is an IgM, k.

Thus, the development and use of hybridoma monoclonal antibodies in this laboratory has proven to be extremely useful in delineating immunoregulatory mononuclear cell subsets as well as defining ontogenic and differentiation markers of normal and malignantly transformed cells.

4) Use of hybridoma antibodies to characterize distinct and overlapping subsets of cytotoxic human lymphocytes.

Mixed lymphocyte reaction (MLR)-activated T cells were analyzed according to the expression of various cell surface markers by the specific cytotoxic T lymphocytes (CTL) generated in the MLR. CTL were found exclusively in a population of MLR-activated T cells that lacked detectable FcγR but that expressed a surface antigen recognized by the 4F$_2$ monoclonal antibody. In contrast, CTL were found in both the Ia-positive and Ia-negative cells after MLR activation. Thus, the specific CTL generated in the allogeneic MLR can be identified and isolated by virtue of the expression of a particular cell surface marker.

In addition, the phenotypes of the various mixed leukocyte culture-activated T cell populations which mediate cytotoxicity in different effector cell assays (cytolytic lymphocyte (CTL), natural killer (NK), and antibody-dependent cellular cytotoxicity (ADCC)) were studied. CTL mediating specific lysis of target cells bearing the stimulating alloantigens could clearly be distinguished from cells mediating ADCC in that CTL were FcγR$^-$ while cells mediating ADCC were FcγR$^+$. In contrast, MLC-generated NK cells were distributed in both the FcγR$^+$ and FcγR$^-$ cell fractions. The presence or absence of Ia antigens did not correlate with any of the cytolytic activities studied. In addition, FcγR$^-$ and not FcγR$^+$ cells responded in the secondary MLC. Thus, although FcγR$^+$ cells generated in the allogeneic MLC play little, if any, direct role in specific cytotoxicity, they nonetheless are generated in large numbers by the MLC. However, their ability following MLC generation to mediate NK and ADCC, both of which may contribute to *in vitro* and *in vivo* lysis of allogeneic cells, might explain their appearance following allogeneic stimulation.

5) Development of B cell hybridomas secreting monoclonal human antibody.

One of the most exciting advances in the laboratory was the successful establishment of a mouse-human heterohybridoma which is secreting pure human monoclonal anti-KLH antibody. Utilizing the antigen specific system which we had developed to trigger human B lymphocytes, we took individuals who had been immunized *in vivo* with the soluble antigen KLH. At an appropriate time following immunization, the individuals' B cells were fused by the standard Milstein-Kohler techniques and a successful heterohybridoma was established secreting large amounts of human anti-KLH antibody. The significance of this from a biomedical and clinical standpoint is extraordinary. We are now working on establishing the same sort of monoclonal antibodies in individuals who have been immunized with hepetitis B vaccine, with pneumococcal vaccine, as well as a number of other relevant microbial antigens. The ability to establish a line secreting unlimited quantities of relevant human antibodies will have major implications in the therapy of infectious diseases as well as in a number of disease states characterized by deficiency of appropriate antibody responses.

In addition, this anti-KLH antibody which surely is of a single idiotype expressed some very interesting *in vitro* functions. When the antibody was added cultures of KLH reactive B and T cells, it was found to be strongly mitogenic. The mechanisms for this is likely a idiotype-anti-idiotype reaction responsible for the triggering of a mitogenic effect. Studies along this line are currently being intensively pursued.

B) Soluble antigen-specific human T cell clones.

1) Development of human antigen-specific T cell clones.

Adapting the methodology of R. Schwartz and B. Sredni who developed soluble antigen-specific T cell clones in the mouse model, we have successfully produced the first antigen-specific T cell clones in man and have perpetuated these with several sub-clonings. They are truly clones by the strict definition and have thus far been demonstrated to be antigen-specific, genetically restricted by HLA-DR with regard to the absolute requirement for presenting cells, and are not alloreactive. The implications of developing antigen specific T cell clones are enormous. In addition to providing truly "pure" T cell populations for functional assay, it provides a source of T cell determinants without contamination from other cell types and other T cell subsets.

Thus, the pressing question of the nature of the T cell receptor can now be approached as well as other questions of the relationship between the expression of surface antigens and functional capabilities. In addition, since these cells are fully characterized with regard to HLA and DR, a whole host of studies relating to the genetics of human immune reactivity can be approached from an entirely new and potentially productive vantage point.

2) Phenotypic and functional characterization of human antigen-specific T cell clones.

Following a number of subclonings, we have characterized the KLH specific human T cell clone according to surface antigen phenotype employing various receptor bindings and monoclonal antibodies directed against cell surface antigens. The T cell clone was shown to be $OKT3^+$, $OKT4^-$ and $OKT8^+$, thus expressing the cytotoxic/suppressor cell phenotype. In addition, we found it to bind to sheep red cells, express Ia antigens, lack Fc receptors for IgG and IgM. In addition, they express the "activation" antigen 4F2.

In several functional studies of co-culture of the clones with KLH immune B cells, it was shown that small numbers (10^3 cells) completely suppress the KLH induced specific anti-KLH response in the presence of antigen without suppressing the polyclonal Ig response of the B cells in culture. As greater numbers of the suppressor clone were added to culture (10^4 and 10^5 cells), both antigen specific as well as polyclonal Ig responses were suppressed suggesting both a specific and non-specific component to the suppressor mechanism.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00212-01 LIR

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Study of the Immunopathogenic Features of Immune Mediated Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Paul Katz, M.D. University of Florida Medical Center
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COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Immunoregulation

SECTION

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A wide range of clinical and pathophysiologic studies were performed in a large number of patients with the idiopathic hypereosinophilic syndrome (HES). Mechanisms of organ system damage and dysfunction were delineated predominantly regarding the endocardiomyopathy of the HES. In vitro stem cell studies delineated the heterogeneous levels of abnormalities in patients with HES. Aggressive medical and surgical approach to the heart disease as well as chemotherapeutic regimens has led to dramatic improvement in prognosis in HES. Various abnormalities at the B and/or T cell levels were precisely delineated in patients with Sjogren's syndrome, SLE, Hashimoto's thyroiditis. An isolated defect in NK cells was described in patients with the Chediak-Higashi syndrome constituting the first description of a primary human immunodeficiency disease with an isolated defect in NK cells. The effect of cyclophosphamide on NK function in patients was studied and demonstrated a marked suppression of function, potentially explaining the occurrence of lymphoproliferative diseases in patients receiving cytotoxic agents. Normal individuals with the HLA-B8, DRw3 haplotype were shown to have abnormalities of RES function as well as defects in subsets of T cells and spontaneous secretion of Ig by B cells.

PHS-6040
(Rev. 2-81)

Objectives:

This laboratory has been involved in studying the clinical, immunopathogenic, and therapeutic aspects of a number of immune mediated diseases. The largest and most intensive effort in this regard has been placed in the area of the study of the spectrum of vasculitis. The details of this particular project will be described in another section. However, employing the methodologies and immunological approaches described in previous sections, we have also been engaged in studying the immunopathogenic features of a number of immune mediated diseases other than the spectrum of vasculitis. Most of these diseases are characterized by abnormalities of immune function or are mediated at least in part by immune mechanisms. The aims of the studies described below were to delineate in a precise manner the immunopathogenic mechanisms in these disease states and to utilize certain of the advances in immunological technology in the investigation of such disorders. These technologies include the in vitro antigen-induced, antigen-specific activation of human B lymphocytes, the utilization of assays for cytotoxic capability of lymphoid cells, the entire array of hybridoma and cloning technology, the precise mechanism of fractionation and identification of human lymphoid cells, as well as a number of other immunologically directed approaches. One of the most intensively investigated diseases has been systemic lupus erythematosus (SLE). This laboratory has been intensively involved in the study of the mechanisms of immune aberration in patients with SLE. We had been among the first to describe the abnormalities of B cell activation as well as certain of the imbalances in immunoregulatory lymphocyte subsets in SLE. Over the past year, the objective has been to more precisely delineate the abnormalities of B cell activation and immunoregulation utilizing our newly described antigen-induced antigen specific in vitro B cell system. In addition, a number of the hybridoma antibodies were utilized to delineate abnormalities of lymphocyte subsets.

Another major thrust in the laboratory was to attempt to establish in vitro antigen-specific system to delineate the mechanisms of triggering and immunoregulation of B cell responses to the hepatitis B antigen in patients with normal response to the infection, in patients with chronic active hepatitis, and in patients who developed hepatitis B antigenemia vasculitis.

Another disease which was studied in some detail was Hashimoto's thyroiditis. These patients have the abnormal production of autoantibodies against thyroglobulin (Tg). Again, we have utilized the experience gained in developing in vitro antigen specific assays of B cell function to dissect out the mechanisms of aberration of B cell function in this particular disease.

A whole host of immunodeficiency diseases were approached in order to delineate the mechanisms of abnormal immunoregulation as well as the primary abnormalities of lymphoid function in these diseases. We have studied a number of the common immunodeficiencies such as common variable immunodeficiency (CVID). In addition, we have stuided the Wiscott-Alrich syndrome utilizing a newly developed G6PD isoenzyme analysis of patients' cells in order to determine the precise level of the genetic defect in this disease.

Finally, a major effort was placed in the delineation of the clinical pathologic, pathophysiologic, and immunologic aspects of the idiopathic hypereosinophilic syndrome (HES). The idiopathic HES is a most unusual disease which this laboratory has been studying for the past ten years. It is characterized by high levels of circulating eosinophils for prolonged periods of time without any recognizible underlying cause for the hypereosinophilia. Of particular note is the fact that these patients have severe organ system dysfunction, particularly endocardiomyopathy. Attempts were made to dissect out the precise mechanism of damage to the endocardium as well as to delineate certain of the other abnormal physiologic events such as the apparent predisposition to thrombotic phenomena.

Methods Employed:

1) The methodology employed in the study of the immunopathologic features of the above mentioned immune-mediated diseases were essentially those which had been worked out within this laboratory to determine the precise mechanisms of lymphocyte activation and immunoregulation in normal individuals. We refer specifically to systems of in vitro culturing of human lymphocytes. Most importantly, the system which was developed in this laboratory for the antigen-induced, antigen-specific triggering of human B lymphocytes and the delineation of their immunoregulation. The antigens used were keyhole limpet hemocyanin (KLH) and tetanus toxoid (TT). In addition, cultures were stimulated with a number of polyclonal B cell activators, most commonly pokeweed mitogen (PWM). In addition to this, the commonly employed blastogenic responses to various antigens and mitogens were employed.

2) A wide variety of cell separation techniques were employed in order to obtain highly purified populations of lymphoid cells. These included plate adherence, purification over G-10 sephadex, elutriation technology to obtain highly purified populations of monocytes, nylon wool column purification, as well as a whole host of positive and negative selection methodologies using hetero-antisera directed against cell surface antigens. In addition, a whole host of commercially available hybridoma antibodies as well as monoclonal antibodies developed in this laboratory were employed to recognize and/or fractionate highly purified populations of human lymphoid cells.

3) Knowledge and experience gained in developing the antigen-induced, antigen-specific B cell system for KLH and TT proved invaluable in attempts at developing an in vitro antigen-specific system to trigger human B cells with the hepatitis B surface antigen. A number of technicologic difficulties have been encountered in this particular system predominantly due to the difficulty in obtaining patients during the period of time of their infection when the circulating B cell repretoire contains sufficient numbers of B cells which spontaneously secrete hepatitis B antibody as well as those which were capable of being triggered by specific antigen or mitogen to secrete hepatitis antibody.

4) Other disease states were approached utilizing the system for measuring human B cells. Specifically, methodologies were established in patients with Hashimoto's thyroiditis to trigger their B cells with specific Tg antigen and measure specific antibody responses to the antigen as well as the induction of polyclonal Ig production following antigenic stimulation.

Disease states such as the Wiscott-Aldrich syndrome were approached with regard to a newly developed technology. We utilized the isoenzyme analysis in the double heterozygote state in order to determine the level of the genetic defect within this disease.

5) A whole range of methodologies within the laboratory and in association with collaborative studies were employed in the investigation of the idiopathic HES. Methods for separating highly purified populations of eosinophils were employed. Leukopheresis of patients' peripheral blood cells resulted in acquisition of large numbers of eosinophils which were used in a number of subsequent studies. From a clinical standpoint, a number of innovative diagnostic tests were performed such as endomyocardial biopsies on patients with the endomyocardiopathy of the HES. These tissues were analyzed using electron microscopy. In addition, echocardiography, angiography, as well as a number of invasive and noninvasive techniques were utilized to delineate the extent of organ system involvement in this perplexing disease state.

A number of precise physiological investigations were performed to determine the nature of the intracytoplasmic granules of the eosinophils. This was done in order to determine the role, if any, of these unique substances in the tissue damage seen in this particular disease. In addition, the mechanisms of the thromboembolic phenomena and the hypercoagulable state in this disease were investigated. A number of *in vitro* studies utilizing culture of stem cells were performed in order to determine the level of abnormality of proliferation and production of eosinophils. Finally, from a clinical standpoint, therapeutic approaches utilizing cytotoxic agents and corticosteroids were employed in the treatment of these patients. Aggressive surgical approaches such as replacement of the damaged atrial-ventricular valves were evaluated.

Major Findings and Significance in Biomedical Research and the Program of the Institute:

A) Studies in the idiopathic hypereosinophilic syndrome (HES).

1) Clinical studies.

Several years ago this laboratory was responsible for delineating the precise criteria and clinico-pathologic features of this unusual and most interesting syndrome. Over the past year we have extended these clinical, pathologic and therapeutic studies. We have now accumulated 50 patients with the idiopathic HES, thus constituting the largest collection of these patients in the world. We have studied the various organ system dysfunction and the mechanisms thereof. From a strictly clinical standpoint, we have continued to utilize our therapeutic protocols of corticosteroids with or without the cytotoxic agent hydroxyurea. This approach together with the other medical and surgical approaches mentioned below have resulted in a marked improvement in the survival and prognosis of this patient population whose morbidity and mortality were extremely high prior to the institution of this therapeutic protocol by this laboratory. In addition to the therapeutic approach utilizing chemotherapy, precise attention was payed to close monitoring and treatment of the cardiovascular and thromboembolic phenomena associated with this disease. In addition, we took the heroic and aggressive step of replacing both the mitral and tricuspid valves in three patients who had the severe restrictive cardiomyopathy of HES superimposed upon incompetent atrio-ventricular valves secondary to involvement of the chordae tendineae of the respective valves with thrombosis and fibrosis secondary to the underlying endocardial disease. The results were striking in that all three patients transformed from cardiac class IV to class I. This aggressive approach in patients with an underlying restrictive cardiomyopathy together with a severe hematologic disease has proven that the aggressive surgical approach to patients with a medical disease which can be reasonably controlled on chemotherapy adds a great deal to the longevity as well as the quality of life of these patients. This particular approach is now being widely recognized as a major advance in the total treatment of this patient population.

Since the heart disease in HES is the organ system involvement which accounts for a major portion of the morbidity and mortality in this disease, attempts were made to identify factors which might be associated with the heart disease in HES. Those patients who developed heart disease were shown to have distinct features which clearly distinguished them from those who were spared from the endocardiofibrosis. These features include: splenomegaly, embolism to the central nervous system (CNS), preponderance of the HLA-BW44 haplotype, thrombocytopenia, elevated B_{12} levels, abnormal bone marrow morphology, abnormal eosinophil morphology, as well as a number of other features. On the other hand, those patients who were spared of the heart disease tended to have an illness that was characterized by features suggestive of an immunologic abnormality. These features include: angioedema, elevated IgE levels, elevated Ig levels, circulating immune complexes, as well as a tendency to be highly sensitive in their response to prednisone. Finally, the patients who developed heart diseases tended to be males with almost all the females belonging in the group who have not developed heart disease.

2) Basic mechanisms of disease activity.

The precise pathophysiologic mechanisms whereby tissue damage occurs in the HES is still unclear at the present time. However, a number of studies performed in this laboratory in collaboration with Dr. Gerald Gleich of the Mayo Clinic have shown that a major protein component of the cytoplasm of the eosinophil, i.e. major basic protein (MBP), is capable of inflicting severe damage on endothelial tissue in culture. Levels of MBP demonstrated in the serum of our patients were equivalent to those levels which induced disruption of endothelial cells in *in vitro* studies. In addition, a direct correlation was demonstrated between the levels of circulating MBP in our patients and the presence of endomyocardial disease. In addition, degranulation of eosinophils was correlated with the presence of overt heart disease. Thus, it is highly suspected that the eosinophils in the idiopathic HES degranulate and release their MBP into the serum which under the high concentrations seen in the intraventricular chamber of the heart results in damage to the endothelium. Subsequent to this, there is the laying down of platelet thrombi with the ultimate propagation of mural thrombi in the chamber of the heart. This then leads to further restriction of the endocardium leading to a restrictive cardiomyopathy. In addition, embolic phenomena occur related to the laying down of thrombi along the wall of the ventricle. With regard to the thrombi in this disease, it has been demonstrated by *in vitro* studies that the patients with the HES tend to have a hypercoagulable state possibly related to the release of eosinophil cationic protein (ECP) into the circulation. Again, with regard to the thrombi, it is this mechanism which engulfs the chordae tendineae of the artrio-ventricular valves leading to valvular incompetence.

A series of stem cell culture techniques utilizing soft agar culture of patients' peripheral blood mononuclear cells have revealed extremely interesting information regarding the underlying pathogenic mechanisms of disease. Several HES patients manifested normal proportions of eosinophils in these culture systems and cannot be differentiated from the normal controls in any of the assays thus far attempted. These patients are those which tend not to have heart disease or tend to be in a quiescent phase of their disease. The remaining patients who manifest an abnormal proportion of eosinophils produced *in vitro* tend to develop overt signs of HES heart disease and the associated clinical syndrome. All of these patients, with the exception of one, showed an increase in proportion of eosinophil colonies formed *in vitro*. Even in these patients, some of whom have clearcut myeloproliferative syndromes, there was evidence that the eosinophilia was indeed a secondary phenomenon since feeder layers from these patients induced an increased proportion of eosinophils in the stem cell cultures from normal individuals. Furthermore, stem cell cultures also revealed that certain individuals with the HES formed colonies which were composed of a number of cellular components including eosinophils, neutrophils, macrophages, and erythrocytes precursors. Thus, these apparently pluripotential progenitor stem cells differ substantially from those described in the literature for normal individuals and constitute a surprisingly unexpected finding in these patients.

B) Studies in patients with Hashimoto's thyroiditis.

Utilizing the newly developed system of antigen-induced, antigen-specific B cell responses for human *in vitro* cultures, we have studied a number of patients with Hashimoto's thyroiditis and have made several interesting observations. Hashimoto's thyroiditis is an organ specific autoimmune disease whereby patients make abnormal amounts of antibody to thyroglobulin (TG). The precise mechanisms whereby this occurs are unclear at present. We have succeeded in establishing the first *in vitro* assay to stimulate the lymphoid cells of patients with this disease with TG and to demonstrate the highly abnormal increased production of antibody *in vitro* to TG. Under the appropriate culture conditions, it was demonstrated that these patients not only have high levels of TG-specific B cells in their circulating B cell repertoire, but also are capable of being triggered nonspecifically with PWM as well as specifically with TG to produce high levels of anti-TG antibody. This degree of antibody production was not seen in normal individuals, and there was a strict correlation between the titers of anti-TG antibody in the serum and the ability to be triggered *in vitro* with antigen to produce anti-TG antibody. Studies are currently underway to determine the state of activation of these B cells as well as the immunoregulatory abnormalities, if any, at the T cell level in this interesting group of patients. Of note is the fact that this is the first demonstration in an organ specific autoimmune disease of the direct triggering of B cells with the relevant antigen in question resulting in the production of antibody against a self determinant.

C) Studies in Sjogren's syndrome and SLE.

1) Sjogren's syndrome.

This laboratory has been involved in the delineation of the abnormalities of B cell triggering in Sjogren's syndrome for some years. We had previously demonstrated that patients with Sjogren's syndrome have a localized activation of B cells in their lip biopsies without the expression of polyclonally triggered B cells in either the peripheral blood or the bone marrow as has been demonstrated in patients with SLE. Thus, it is now generally felt that Sjogren's syndrome is a localized hyperreactivity of B cells without the severe B cell and T cell abnormality seen in patients with SLE. These studies have been done predominantly using the polyclonal system. However, most recent studies utilizing the antigen specific system have demonstrated distinct abnormalities in patients with Sjogren's syndrome at the B cell level. In addition, a number of patients with Sjogren's syndrome were studied in regard to the abnormalities of NK function and it was found that obvious abnormalities were not present.

2) Studies in SLE.

We had previously been among the first laboratories to demonstrate abnormalities at the B cell level in patients with SLE as well as aberrancies at the level of T cell cell subsets controlling B cell function. These studies have been extended using the antigen-specific system and it has been found that SLE patients have abnormalities at multiple levels of B cell triggering when one utilizes an antigen-specific system. These studies are still at the preliminary phase. However, utilization of the antigen-specific system with the advantages of being able to measure the control of antigen-specific responses as well as the overlap between antigen specificity and polyclonality will lead to important observations in the pathophysiologic mechanisms in SLE. In fact, one of the major motivations in establishing the antigen-specific system was to dissect out this interesting and perplexing dichotomy and overlap between polyclonality and antigen specificity. The SLE model may yield fruitful information in this regard since it has been well established by our laboratory and other laboratories that the B cells in patients with SLE are polyclonally triggered and one of the possibilities of the mechanisms of this polyclonal triggering is an aberrant polyclonal response to a specific antigenic stimulus.

D) Studies of cytotoxic capability in disease states.

1) The Chediak-Higashi syndrome.

We have made an extremely interesting observation in this laboratory in demonstrating that patients with the Chediak-Higashi syndrome (CHS) have an isolated deficiency of NK cells. This is the first description in man of a primary immunodeficiency disease with an isolated defect in NK cells. This was shown to be truly a functional defect since the abnormality was not due to a lack of effector cells or an inability of effector cells to bind normally to target cells. Furthermore, the defect was partially, but not completely corrected, in vitro with interferon. In addition, the defect was corrected by the addition of cyclic nucleotides to cultures. Current studies are underway to determine the precise level of the defect in NK in the CHS with regard to binding of NK cells to target cells, recycling of NK cells, as well as the precise mechanisms of functional defect at the killing level.

2) Effect of cytotoxic agents on NK function.

In a series of studies which were superimposed upon our study of the role of cyclophosphamide in B cell triggering as well as clinically in various disease states, investigations were aimed at determining the effect, if any, of cyclophosphamide on NK function. Since NK function is felt to play a role in immune surveillance against neoplastic transformation, particularly lymphoproliferative diseases, it was particularly relevant to determine if patients receiving cyclophosphamide would have a defect in NK cells related to their drug regimen. Indeed, it was found that patients receiving cyclophosphamide had a defect at the level of NK cells. This may help to explain the neoplastic transformation seen in certain patient populations who are treated with cyclophosphamide.

E) Role of HLA in normal immune responses.

In a series of experiments aimed at determining the relationship between HLA markers and integrity or lack thereof of reticuloendothelial system (RES) function in patients with dermatitis herpetiformis (DH), extremely interesting results were found with regard to normal individuals. It was shown that patients with DH had markedly abnormal RES clearances. Since virtually all patients with DH are of the HLA-B8, DRw3 haplotype, the control population for the study were groups of normal individuals randomly selected as well as those with the HLA-B8, DRw3 haplotype. It was found that perfectly normal individuals who were of the HLA-B8, DRw3 haplotype showed abnormalities of RES clearance. In addition, these individuals had significantly decreased numbers of lymphocytes which expressed Fc receptors for IgG. Moreover, they had significantly elevated numbers of B cells which spontaneously secreted Ig in culture. These findings are of potentially great importance in appreciating the role of the HLA-B8, DRw3 haplotype in immune mediated diseases since this haplotype is seen to a significantly high degree in several autoimmune diseases.

F) Studies on the level of defect in the Wiscott-Aldrich syndrome.

G6PD isoenzyme analysis in the double heterozygote state has shown that the genetic defect in this disease separately and independently affects platelets and T lymphocytes in all pedigrees studied. This has led to the conclusion that the thrombocytopenia seen in this disorder is a direct consequence of the genetic defect. In addition, it was demonstrated that any peripheral blood cell type could demonstrate allelic exclusion, but that this phenomenon varied between individual carriers.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00213-01 LIR

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Clinical, Immunopathogenic and Therapeutic Studies in the Spectrum of Vasculitis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Anthony S. Fauci, M.D., Chief, LIR/NIAID

Other Investigators:

Thomas R. Cupps, M.D., Medical Officer, LIR/NIAID
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Joseph E. Parrillo, M.D., Chief, MICU, NIH Clinical Center

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Immunoregulation

SECTION

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The LIR is now prospectively studying the largest group of patients with the vasculitic syndromes of any center in the world. Clinical, pathophysiologic, immunopathogenic and therapeutic results obtained over the past 13 years have allowed us to design a revised categorization scheme for the vasculitides which has now reached world wide acceptance. We have instituted aggressive chemotherapeutic regimens in several, formerly universally fatal diseases such as Wegener's granulomatosis and have induced cures in 90% or more of these patients. We have now applied these approaches with remarkable success to other of the vasculitic syndromes including systemic vasculitis of the polyarteritis nodosa group, isolated CNS vasculitis, Takayasu's arteritis, and recurrent panniculitis with vasculitis. We have established associations between several of these diseases and various HLA-DR haplotypes. These patient populations have been utilized to precisely delineate aberrations of activation and immunoregulation of lymphoid cell function and have served as excellent models for the concomitant study of normal human immune system. In addition, the precise effects of various therapeutic regimens, particularly corticosteroids and cytotoxic agents, on human lymphoid cell activation and immunoregulation have been described.

Objectives:

This laboratory has been intensively involved in the study of the clinical, immunopathogenic and therapeutic aspects of the entire spectrum of vasculitis. A major effort has gone into intensive study of in order to redefine a more appropriate classification scheme with the ultimate goal in mind of establishing optimal therapeutic regimens for the various subcategories of the vasculitides. This would entail intensively working up from a clinical and pathophysiologic standpoint a wide variety of patients falling under the broad spectrum of vasculitis. In this regard, a large number of patients with the vasculitic syndromes have been admitted over years to the NIH Clinical Center to undergo these clinical study protocols. The basic objectives of the study were as follows: 1) to classify and categorize more appropriately and more precisely the various clinical syndromes within the spectrum of vasculitis, 2) to identify where possible pathophysiologic mechanisms and events associated with the expression of the disease state, 3) to design and implement therapeutic regimens for the various categories of the vasculitides, and 4) to use the patient population as models of aberrancies of immune function in order to supplement the studies of the activation and immunoregulation of human lymphocyte function which are a major component of the endeavors of the laboratory.

Methods Employed:

1) To admit large numbers of patients within the category of the vasculitides and to work them up according to standard clinical and pathological approaches.

2) To study from an immunopathogenic standpoint the immune abberrancies in these patients particularly in the *in vitro* antigen specific and polyclonal B cell functional systems which have been established and are under current study within the laboratory.

3) To devise improved methods of diagnosis and monitoring of disease activity.

4) To determine any genetic associations within the various subcategories of disease states.

5) To design, implement and follow-up on various therapeutic regimens utilized in these particular diseases.

6) To employ various procedures such as leukopheresis to obtain large numbers of peripheral blood lymphoid cells for study.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

A) Categorization of the vasculitic syndromes.

Over the past 13 years, a large number of patients with the various vasculitic syndromes were admitted to the NIH Clinical Center under the direction of Dr. Fauci. These patients were followed extremely carefully from a clinical standpoint over a number of years with the purpose in mind to obtain a prospective approach towards studying the evolution and natural cause of the various subcategories of diseases. Over the past year, this effort has been even further intensified such that the laboratory is currently following the largest group of patients with the vasculitic syndromes of any medical center in the world. We have now established (and it is generally accepted worldwide) a revised categorization of the vasculitic syndromes based on the relative degree of systemic versus cutaneous involvement as well as the establishment of more precise criteria for distribution of the various patient populations into defined subcategories. Major review articles as well as the results of prospective studies have been reported which are now utilized by most medical centers throughout the country and the world.

B) Pathogenesis of the Vasculitic Syndromes.

A number of studies have been performed throughout the years and most intensively over the past year delineating the role of circulating and deposited immune complexes in the initiation and perpetuation of the vasculitic syndromes. In addition to that, various exciting studies were performed in which the relationship between HLA-B8 and HLA-DRw3 in normal individuals was associated with abnormalities of immune function and immunoregulation particularly at the level of the reticuloendothelial system. It had been noted that patients who manifest the HLA-B8 and HLA-DRw3 haplotype had a propensity toward immunopathic diseases. This was seen in a number of our patients and HLA-B8 haplotype association was in fact described by us to be predominant in patients with Wegener's granulomatosis. By extending these studies, we in association with Drs. Thomas Lawley and Michael Frank established that the HLA-B8 and HLA-DRw3 haplotype in normal individuals was in fact associated with a hyperreactivity at the B cell level, a deficiency of an immunoregulatory T cell subset, as well as a significant defect in RES clearance.

In addition, an extensive series of studies in Cogan's syndrome delineated two major categories: a) classic Cogan's syndrome, and b) atypical Cogan's syndrome. The relative degree of vasculitic component in each of these was delineated and has led to a more precise understanding of the association of Cogan's syndrome-like symptoms in various vasculitic syndromes as well as a precise delineation of the degree of true vasculitis in classic Cogan's syndrome.

Over the past year a series of studies were initiated to delineate the true categorization and pathogenesis of what was formerly called Weber-Christian disease. This disease has also been known as chronic relapsing panniculitis. We have found by prospectively studying a moderately large number of patients that indeed the clinical manifestations are much more diverse and in certain respects serious than was previously thought. The vasculitic component of Weber-Christian disease has now been recognized and indeed is a major component of a certain subset of these patients. This has prompted us to initiate in these patients the type of therapy which will be described below and which has been used successfully in certain of the other vasculitic syndromes.

Over the past year, we have admitted a number of patients with isolated central nervous system vasculitis. This is an extremely poorly understood syndrome characterized by severe vasculitis of the central nervous system leading to marked incapacity and death in a high percentage of patients. This disease has not been perspectively studied to any great extent prior to our initiation of these ongoing studies. It has been found that in most cases these patients have disease activity limited to the central nervous system without the usual expression of systemic signs, symptoms, and parameters of disease. Again, this patient group has been entered into a prospective therapeutic protocol study which will be described below. One of the major advantages of a study of this sort is to precisely delineate the full clinico-pathologic features and manifestations of this disease since it is poorly recognized and associated with a great deal of confusion in the clinical and therapeutic approach which physicians have towards this perplexing syndrome.

An exciting finding related to the genetic studies in these protocols has been the finding that 100% of our patients with Takayasu's arteritis (aortic arch syndrome) express the B cell antigen termed MB3. Seven out of ten patients were found to have the DRW4 haplotype in their HLA complex. This is far in excess of the random associations in the general population. Thus, the strong association between disease and an immune response gene product points to a definite genetic component in this disease, closely linked to the immune response gene in normal humans.

C) Treatment of the Vasculitic Syndromes.

Several years ago this laboratory was the first group to establish the remarkable efficacy of the use of cytotoxic agents, particularly cyclophosphamide, in the treatment of non-neoplastic, immune-mediated diseases with a vasculitic component. The disease in question was Wegener's granulomatosis which was invariably fatal prior to the institution of cytotoxic therapy. Over the past 13 years, we have prospectively treated over 90 patients with a combination of 2 mg/kg/day of cyclophosphamide together with alternate day corticosteroid therapy. The disease went from being essentially uniformly fatal to being virtually curable with remission and cure rates seen in greater than 90-95% of our patients. Over the past year, we have continued the studies with Wegener's granulomatosis, but have intensified our efforts to apply this remarkably beneficial therapeutic regimen to a number of other vasculitic syndromes. In this regard, it is extremely important to point out why the appropriate categorization of the vasculitides mentioned above was carried out. There are certain of the vasculitides which have a waxing and waning course and which can spontaneously resolve without serious consequences. Therefore, it is essential to identify those syndromes whose course would be unrelenting without treatment and whose prognosis would be extremely poor if appropriate therapeutic intervention was not introduced. Thus, the combination of our efforts at categorization as well as our experience with the therapeutic protocols has led us to establish strict and efficacious guidelines to the therapeutic approach towards the vasculitides. In this regard, we have now studied a large number of patients with severe systemic vasculitis of the polyarteritis nodosa group. We have clearly demonstrated that in that subcategory of patients who would otherwise have fulminant disease, the use of cyclophosphamide together with alternate day corticosteroids is remarkably efficacious in suppressing disease activity. Indeed, we have studied a large number of patients and have shown that remission and cure rates can be induced in a substantial proportion of these patients.

Because of the successes in the Wegener's granulomatosis and polyarteritis nodosa groups, we have introduced this therapeutic approach in a number of other of the vasculitides. Two of these in which favorable results have been found are Takayasu's arteritis and the vasculitic subgroup of recurrent panniculitis. With regard to Takayasu's arteritis, we now are following a large number of patients, one of the largest groups in the world, and have noted favorable results with this therapeutic regimen. In addition, working with the vascular surgeons from the National Cancer Institute, we have taken a somewhat aggressive approach towards bypassing involved vessels of the aortic arch and its branches. Thus, not only have we been successful in suppressing the inflammatory component of the disease, but by the use of reconstructive surgery, we have preserved and returned function to the limbs and organs which were otherwise markedly compromised.

Studies of recurrent panniculitis (Weber-Christian) are in their incipient stage. However, we have noted several patients who are refractory to any type of therapy including high dose corticosteroids, who have acutally gone into remarkable remission with the introduction of cytotoxic agents such as cyclophosphamide.

Thus, this year has witnessed an acceleration of our studies on the clinical, pathogeneic and therapeutic aspects of the vasculitic syndromes and has yielded findings and results which are most gratifying in that we have now firmly established guidelines both for categorization as well as therapy of these not uncommon and serious group of diseases. Our classification schemes and therapeutic protocols have now been adapted by medical centerd throughout the country and the world, and our results are beginning to be firmly corroborated by numerous other investigators.

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I N D E X

LABORATORY OF INFECTIOUS DISEASES

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SUMMARY STATEMENT -- Annual Report of the

Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
October 1, 1980 to September 30, 1981

The main focus of the research programs of LID continues to be the natural history and prevention of the three major uncontrolled, acute infectious diseases of man--acute respiratory tract disease, viral hepatitis, and acute gastroenteritis.

Our understanding of the etiology and epidemiology of viral respiratory and gastrointestinal tract diseases far exceeds our capacity to control or to alter the course of these illnesses. Effective immunoprophylaxis represents a formidable challenge. The obstacles that impede progress are diverse and may affect almost any stage of vaccine development. For example, a number of viral pathogens cannot be grown in tissue culture, and for this reason efforts at immunoprophylaxis are blocked at the very earliest phase. In other instances serious problems are not encountered until a late stage of vaccine evaluation.

Our approach to immunoprophylaxis is heavily influenced by the major role local immunity plays in resistance to most respiratory viral pathogens. The most effective way to stimulate such immunity is through infection of the respiratory tract. Similarly, local intestinal immunity appears to play a significant role in resistance to rotavirus disease in experimental animals. Furthermore, infection of the respiratory or gastrointestinal tract induces systemic as well as local immunity, and thus all phases of host defense mechanisms are brought into play. For these reasons emphasis has been given to the use of live attenuated viral vaccines for the prevention of respiratory and gastrointestinal viral diseases rather than parenteral administration of nonreplicating antigen. However, one should reserve judgment concerning the usefulness of nonreplicating antigen since in high concentration it may induce effective resistance when administered directly into the respiratory or gastrointestinal tract. Soon it will be possible to evaluate this approach since recombinant DNA technology should allow us to produce virtually unlimited amounts of pure antigen.

Finally, it should be noted that many viruses that infect mucosal surfaces induce an immunity that is both partial and transient. This should not deter us from attempting to develop vaccines for these agents since partial immunity is usually sufficient to diminish the disease manifestations of reinfection to an acceptable level. In the future it may be possible to improve upon naturally acquired immunity by using attenuated mutants to optimize the timing of reinfection.

Enteric Viral Disease

There has been an explosion of new information concerning the viruses that cause acute gastroenteritis. First, the Norwalk virus and its relatives were identified and shown to be etiologic agents in the syndrome of acute, epidemic gastroenteritis. Recently the prototype of this group, the Norwalk virus, was shown by Drs. Greenberg, Singh, et al., to be associated with one-third of 70 unselected outbreaks of acute gastroenteritis. The Norwalk virus-associated outbreaks included several which were waterborne or related to ingestion of oysters. Second, the human rotaviruses are now firmly established as one of the major causes of serious diarrheal disease of infants and young children throughout the world.

Until recently there were no prospects for the development of vaccines directed against the viral enteritides since none of the viruses causing such disease could be cultivated in vitro. This remains true for the Norwalk group of viruses which have resisted all attempts at cultivation in the laboratory. At present the only source of these viruses is the feces of infected individuals.

The control of rotaviral infection in infancy and early childhood represents an important public health challenge since this form of acute gastrointestinal illness accounts for a significant amount of severe pediatric diarrheal disease in most parts of the world. Efforts at immunoprophylaxis have been hampered by the fastidious nature of the recognized human rotavirus serotypes. Except for a mutant of the Wa strain of type 2 virus, human rotaviruses have not been grown serially to high titer in tissue culture. Instead they produce an abortive infection which yields little or no infectious virus.

Recently, in studies by Dr. Wyatt, et al., a tissue culture-adapted mutant of the Wa strain emerged during serial passage of this type 2 virus in gnotobiotic piglets. It appears that a mutant capable of efficient growth in cell culture had been selected during the serial passage in piglets. The tissue culture-adapted strain was shown to be of human origin by polyacrylamide gel analysis of its RNA and by cross-neutralization.

Similar efforts to select a culture adapted mutant of type 1 virus failed since this virus could not be passaged in piglets. For this reason Drs. Greenberg and Wyatt developed another strategy which took into account the segmented viral genome of the rotaviruses that belong to the family Reoviridae. Since other members of this virus family undergo genetic reassortment with high frequency during mixed infection, they attempted to rescue noncultivatable human rotavirus by coinfection with a cultivatable, temperature sensitive (_ts_) mutant of bovine rotavirus.

The initial attempts at gene rescue were performed with a noncultivatable type 2 human virus since the antisera needed to select and identify the desired type 2 viral reassortants could be chosen beforehand based on tests performed with the cultivatable mutant of type 2 virus. The noncultivatable type 2 human rotavirus was inoculated into African green monkey kidney cell cultures which were coinfected with a _ts_ mutant of a cultivatable bovine rotavirus. Selection of the desired viral reassortants was achieved by exposing progeny from the mixed infection to potent, bovine rotavirus-specific antiserum that neutralized bovine rotavirus but not type 2 human rotavirus. Surviving virus was then plaqued at a restrictive temperature (39 C) to eliminate the _ts_ bovine rotavirus parent and _ts_ reassortants. In this manner viral reassortants were recovered that (_1_) grew to high titer in tissue culture, (_2_) produced plaques with high efficiency, and (_3_) were specifically neutralized by antiserum to type 2 virus. Analysis of the RNA segments from these viruses confirmed that reassortment of genes had occurred. At least three genes were derived from the type 2 human rotavirus parent and at least six others from the bovine virus parent.

Following rescue of noncultivatable type 2 human rotavirus, a similar attempt was made by Dr. Greenberg with noncultivatable type 1 virus. This attempt was also successful and yielded cultivatable reassortants that were specifically neutralized by antiserum to type 1 human rotavirus. The rescue technique has now been expanded to include 33 rotavirus containing stool specimens from various countries. Twenty viruses were successfully rescued. These reassortants fell into three distinct serotypes indicating the probable extent of polymorphism of the human rotavirus neutralization protein (Drs. Wyatt, and Greenberg).

Analysis of reassortant genotype by gel electrophoresis or nucleic acid hybridization coupled with immunologic characterization allowed us to determine which gene was always present in viruses that were neutralized by a specific human rotavirus antiserum or that exhibited subgroup reactivity by immune adherence hemagglutination (IAHA) or ELISA. These studies by Drs. Kalica, Kapikian, Greenberg and Flores showed conclusively that the neutralization protein of the type 2 human rotavirus was coded for by the 9th gene, while the subgroup antigen was coded for by the 6th gene. Furthermore, the only gene that was always derived from the cultivatable animal rotavirus parent was the 4th RNA segment indicating that this gene was responsible for restriction of growth of human rotaviruses in tissue culture.

The availability of cultivatable viral reassortants with type 1, type 2 or type 3 human rotavirus specificity should facilitate efforts to develop an effective rotavirus vaccine. The exact form of this vaccine cannot be predicted at this time. Possibly types 1, 2 and 3 antigens will be needed since these serotypes appear to cause most of the currently recognized rotaviral disease of early life. However, recent serologic studies by Dr. Wyatt indicate that infants recognize more shared antigenic determinants on the neutralization protein of these virus serotypes than do experimentally immunized animals suggesting that one virus serotype may provide resistance to other serotypes.

It should be noted that evaluation of a variety of reassortant viruses in humans and experimental animals may shed light on the genetic determinants of virulence. Perhaps we may be able to identify certain mixed constellations of animal and human rotavirus genes that render viral reassortants attenuated for humans. This type of information should prove useful in efforts to develop live attenuated strains of rotavirus for prevention of human disease.

In the meantime, a suspension of the Wa tissue culture adapted mutant was prepared for evaluation in volunteers. After successful completion of safety tests, the African green monkey kidney culture type 2 human rotavirus was administered orally to 12 adult volunteers, two with a high level of serum neutralizing antibody and 10 with a low level of such antibody. None of the volunteers developed a rotavirus specific illness. To date, we have failed to detect rotavirus in the stools of any of the volunteers. Perhaps, the Wa mutant is sufficiently attenuated that it will be highly infectious only for individuals without prior rotavirus experience.

During the past year significant advances were made in our understanding of the transcription and translation of rotavirus RNA genes. Initially Dr. Flores optimized the transcription of (+) single stranded (ss) RNA's from double stranded (ds) genomic RNA's present in viral cores and showed that they represented faithful copies of genomic RNA. These ssRNA's were then used to translate rotavirus proteins in a cell free system and to study the relatedness of corresponding rotavirus genes by nucleic acid hybridization. ssRNA's heavily labelled with ^{32}P were hybridized to melted genomic RNA, treated with S1 nuclease and then analyzed by gel electrophoresis. Complete homology between the ssRNA probe and genomic RNA was indicated by the formation of hybrid RNA segments with a mobility identical to that of genomic dsRNA, whereas probes and genes that were not homologous yielded smaller bands as a result of S1 nuclease digestion of non-homologous regions. This hybridization technique has proved useful in (a) studying the epidemiology of human rotavirus infection, (b) defining the polymorphism of rotavirus genes and (c) comparing a variety of human and animal rotaviruses for evidence of genetic relatedness.

Respiratory Viral Vaccines - Among the respiratory viruses, cultivatability is not a significant problem in vaccine development. Indeed the large number of cultivatable respiratory tract viral pathogens represents one of the major obstacles to successful immunoprophylaxis. The list of cultivatable viruses now known to play a role in respiratory tract disease exceeds 120, and more are likely to be uncovered. However, there is general agreement concerning the priorities for immunoprophylaxis. In decreasing order of importance, the main targets for vaccine development include influenza A and B viruses, respiratory syncytial virus, parainfluenza viruses, certain of the adenoviruses, and the rhinoviruses.

At present the only respiratory viral vaccine available to the general public is inactivated influenza virus vaccine. Abrupt antigenic variation caused by gene transfer, progressive antigenic variation resulting from point mutation, and the phenomenon of "original antigenic sin" each act to diminish the potential effectiveness of inactivated influenza virus vaccine. Antigenic diversity of a different sort has thwarted efforts to develop vaccines against the rhinoviruses, which now include at least 100 distinct serotypes.

Defined Mutations that Specify a Satisfactory Level of Attenuation - Viruses bearing specific, identifiable, attenuating mutations represent the vaccine strains of the future because the genetic basis for attenuation is known and can be monitored directly during all phases of vaccine development, manufacture, and utilization in humans. The greatest success in producing and characterizing such attenuating mutations has been achieved with influenza A virus, whose segmented genome facilitates the segregation and characterization of individual genes. Although inactivated influenza virus vaccines as now formulated are effective, they do not provide complete protection nor do they appear to retain their effectiveness when administered annually. For this reason, there is renewed interest in the development of a live attenuated virus vaccine that would mimic natural infection in its greater, broader, and more durable immunity.

In an extensive effort led by Dr. Murphy, chemical mutagenesis was used to produce *ts* mutations on genes that code for nonsurface viral proteins so that the mutant genes could be transferred into reassortant viruses bearing the surface antigens of new epidemic or pandemic strains. It is implicit in this approach that chemically induced *ts* mutations are primarily responsible for the attenuation of reassortant viruses bearing *ts* genes. This was shown to be the case for reassortant derived from two different *ts* donor viruses. The *ts* genes of the donor viruses were the only genes that were always transferred into ts reassortants that were satisfactorily attenuated for humans. In one instance the only genes transferred from the donor strain were its two ts genes, yet the resulting ts reassortant was satisfactorily attenuated for volunteers who lacked immunity to both viral surface antigens. In other studies (Drs. Massicot and Murphy) it was shown that these ts genes were responsible for restriction of growth of ts reassortants in the respiratory tract of the hamster.

The most satisfactory ts donor developed by Dr. Murphy is the influenza A/Udorn/72 (H3N2) ts-1A2 virus that possesses ts mutations on the genes coding for the P1 and P3 polymerase proteins. These proteins are involved in transcription of complementary RNA from viron RNA, which is the earliest phase of viral replication. Transfer of the two ts genes from this donor into 11 different reassortants yielded viruses with marked temperature sensitivity as indicated by a 37°C shutoff temperature for plaque formation. These reassortants exhibited a 10,000-fold suppression of viral replication in the lungs of hamsters and a 100-fold restriction of viral growth in the nasal turbinates. Furthermore, the reassortants were stable genetically during viral replication in hamsters, and infection induced resistance in hamsters to challenge with wild-type virulent virus.

When tested in susceptible adults 3 different ts-1A2 reassortants were suitably attenuated, stable, and antigenic. Unfortunately one of the ts-1A2 reassortants exhibited genetic instability during an asymptomatic infection of a susceptible child. The mechanism for loss of the ts phenotype was quite complex and involved intragenic mutations on the ts P1 and P3 genes as well as "suppressor" or second site mutation on another gene (P2) that corrected the temperature sensitivity of the P3 gene product (Drs. Tolpin and Murphy). This type of genetic instability prepresents the central unsolved problem in the ts mutant approach. This is particularly frustrating since the ts-1A2 reassortants are satisfactorily attenuated for highly susceptible individuals.

Strategy for Producing More Stable, Defined Mutations in Negative Strand RNA Viruses, Such as Influenza Virus

It is clear that one of the most urgent needs in experimental immunoprophylaxis is the development of attenuated mutants that are completely stable genetically. The restriction of growth of an attenuated mutant in vivo exerts strong positive selection for new mutations that allow the virus to grow better and escape the limitations imposed by its original mutations. We need stable mutants that are unable to escape from their attenuated phenotype by reversion and/or suppression during replication in completely susceptible individuals.

Theoretically deletion mutations should be stable because they are not subject to reversion and it is unlikely that they would be easily suppressed by a new mutation at another site on the viral genome. For these reasons we have initiated attempts to produce stable deletion mutations that will render a virus sufficiently defective that it becomes attenuated but not so defective that it loses viability. This type of genetic surgery can only be performed on DNA. Hence those viral genomes that are composed of RNA must be transcribed into DNA and then manipulated in this form. This presents us with the difficult problem of transcribing the altered mutant DNA into an RNA form that can be transferred back into an infectious virus.

Before discussing strategies for producing stable mutants for use in immunoprophylaxis, it should be noted that stable, viable, deletion mutants of several DNA animal viruses (SV40, polyoma and adenovirus) have been prepared by others, and a number of these mutants exhibit a reduced capacity for viral replication. This is of interest since reduced replicative capacity is synonymous with attenuation. Both nonstructural regions and structural genes have been identified as sites in which viable deletions can be produced. For example, at least three regions within the SV40 genome can sustain deletion without loss of viability. Furthermore, deletion within either type of region can reduce replicative capacity of the affected virus. Similar viable deletions have also been produced within the adenovirus genome. Although it may be unwise to generalize to other virus groups, I am encouraged that similar deletion mutants can be prepared for use in immunoprophylaxis of viral respiratory and gastrointestinal tract disease.

The general plan for construction of deletion mutants of RNA viruses was initially developed by Drs. Lai and Markoff, et al., for influenza A virus. The first phase of this plan involved cloning individual influenza RNA gene segments using an Escherichia coli K12 plasmid system. Initially, positive and negative complementary DNA (cDNA) strands were prepared separately by reverse-transcription. The positive strands of cDNA were transcribed from genomic RNA segments by using a specific dodecamer DNA primer with a sequence complementary to the conserved 3'-terminal nucleotides of virion RNA. The negative strands of cDNA were transcribed from cytoplasmic polyadenylated viral messenger RNA (mRNA) segments by using an oligo (dT) primer. The single-stranded DNA molecules from both preparations were annealed to generate double-stranded DNA segments. The in vitro synthesized double-stranded DNA segments were then inserted into plasmid pBR322 at the specific PstI site using the dG-dC tailing technique. The hybrid DNA molecules were then used to transform E. coli, and transformants containing influenza virus gene sequences were identified by hybridization. In this manner, putative full-length influenza virus DNA segments were obtained corresponding to genes coding for the NS proteins (gene 8), the M proteins (gene 7), the neuraminidase (gene 6), the nucleoprotein, i.e., NP (gene 5) and the hemagglutinin (gene 4), . This approach should be generally applicable to other influenza viral genes as well as to other negative strand RNA viruses.

The influenza virus-specific DNA sequences released from the recombinant plasmid by PstI cleavage were characterized by digestion with other restriction enzymes. Restriction enzyme cleavage sites were positioned on the linear map of each DNA segment and oriented with respect to the 3'-terminus of virion RNA.

Sequence analysis of the terminal nucleotides of the majority of cloned viral DNA segments indicated that both termini of virion RNA were fully represented. This suggested that these cloned DNAs were complete and contained all the information present in the viral RNA segments from which they were derived. Thus, RNA transcribed from such cloned DNA should have all of the control signals for viral gene replication and expression in eukaryotic cells. Indeed, three of the cloned genes (NA, M and NS) have been completely sequenced and each appeared to contain the full base sequence of its corresponding RNA segment. The NA cloned DNA has been fully sequenced by Dr. Markoff, while the other two genes were sequenced in collaboration with Dr. Robert Lamb, Rockefeller University.

These complete DNA clones have been used to elucidate several interesting features of influenza virus mRNA transcription. For example, studies utilizing cloned influenza DNA provided conclusive evidence for the transcription of two separate messages from the same RNA segment. This unexpected phenomenon was observed with both the 7th and 8th RNA segments. Also, RNA splicing was first recognized during the course of these studies of transcription of the 7th and 8th RNA segments carried by Dr. Lai in collaboration with Dr. Robert Lamb of Rockefeller University. Previously it had been shown by others that splicing did not occur during transcription of the hemagglutinin gene (4th RNA segment). With both the 7th and 8th influenza A genes, the larger message, coding for the larger protein, was colinear with its gene segment, while the smaller message, coding for a smaller protein, was spliced and transcription continued subsequently in a different reading frame.

The second phase of the plan involves the use of full-length influenza DNA to produce virion RNA in eukaryotic cells. A late region deletion mutant of SV40 was used for construction of influenza virus-SV40 hybrid DNA molecules by Drs. Sveda, Lin and Lai. In the first experiment, influenza hemagglutinin DNA was inserted by Drs. Sveda and Lai into the region of the SV40 genome previously occupied by the deleted sequences that code for SV40 capsid proteins. This type of hybrid DNA molecule was then propagated in eukaryotic cells that were coinfected with an early *ts* mutant of SV40. When cell cultures were incubated at restrictive temperature, the hybrid influenza DNA-SV40 molecule supplied the function defective in the SV40 early *ts* mutant and vice versa, thereby permitting both SV40 molecules to replicate. Replication of influenza DNA-SV40 hybrid molecules in eukaryotic cells led to transcription of viral mRNA from influenza DNA and production of glycosalated hemagglutinin that was expressed on the surface of the infected cell. If vRNA can be transcribed in a similar fashion from influenza DNA inserted into SV40 in the opposite orientation, it may be possible to rescue vRNA from cells by coinfecting with an influenza virus and selecting for reassortant virus particles containing this RNA. In this manner, stable, site-specific mutations, such as deletions, introduced into the cloned DNA by enzyme surgery might be transferred back to influenza virus.

Similar efforts can be undertaken for other negative strand RNA viruses such as the parainfluenza viruses and RS virus. However, these viruses will probably prove technically more difficult to transcribe into DNA because they have a large genome that is not segmented.

An Alternate Strategy

In the end we may find that some viruses are not able to sustain significant deletions without losing their capacity to infect and elicit a satisfactory immune response. In these situations an alternative strategy of the type now being evaluated by Dr. Murphy will be needed. For viruses with a segmented genome, consideration should be given to the use of reassortant viruses containing host range genes derived from animal or avian viruses. Such reassortant viruses should exhibit some degree of growth restriction and attenuation in humans as a result of the inefficient manner in which host range gene products function in human cells. This attenuating effect may be amplified by the incompatability of these proteins with human viral proteins as they interact in multimeric viral complexes. Further, host range genes should be relatively stable genetically since they differ considerably from the corresponding human viral genes and would require many mutational changes before they could produce a product that is not restricted in human cells. Dr. Murphy has identified several avian influenza A viruses that are restricted in their growth in squirrel monkeys. The genetic basis for this restriction and its possible usefulness in immunoprophylaxis will be evaluated through the study of appropriate reassortants produced by mating these avian viruses with virulent human influenza A virus.

Hepatitis A Virus

Isolation of HAV in Tissue Culture - HAV was recently isolated in tissue culture for the first time by Provost. We have attempted to isolate HAV directly from clinical materials into a cell line suitable for vaccine development. These studies, under the direction of Dr. Richard Daemer, have utilized three different strains of HAV: The MS-1 strain studied by Dr. Krugman at Willowbrook, the SD-11 strain recovered by Dr. Dienstag from a Naval recruit in San Diego and the HM-175 strain supplied by Dr. Gust from Australia. Although partially successful results were obtained with all three strains, consistently better results were obtained with the HM-175 strain, and subsequent studies were carried out only with this strain. HAV is currently in 13th passage in primary AGMK, and the duration of each passage has shortened from approximately 8 weeks to 3-4 weeks. Virus isolated directly from feces is growing as well as marmoset-passaged virus. We are currently attempting to further adapt HAV to grow in primary AGMK and to adapt it to growth in diploid cultures. Significantly, the 10th tissue culture passage of HAV appears to be attenuated for chimpanzees.

Cloning of HAV - Because HAV is difficult to grow in tissue culture and because a number of questions remain about its molecular virology and taxonomy, we have attempted to clone the HAV genome in *E. coli*. Dr. John Ticehurst has purified HAV from infected marmoset livers and extracted viral RNA. Several clones of plasmid that contain DNA hybridizeable with the original RNA preparation have been isolated.

Preparation of Monospecific (hybridoma) antibody to HAV - Dr. Vincent McAuliffe has attempted to prepare hybridoma antibodies from mice hyperimmunized with HAV partially purified from marmoset liver or tissue culture. Several hybridoma clones that appear to synthesize antibody to HAV have been detected and are currently being purified and further characterized.

Hepatitis B Virus

HBV Vaccine - Work by Drs. Purcell and McAuliffe in collaboration with Dr. Gerin has continued on the development and characterization of hepatitis B vaccine. Modifications made in the vaccine formulation have resulted in a highly immunogenic preparation, capable of stimulating antibody in a majority of recipients receiving as little as 5 ug of vaccine and producing a rapid and brisk antibody response: 60-80% of vaccinees develop antibody within one month of vaccination and 95% seroconvert by completion of the vaccination series. Plans are underway to carry out an efficacy trial of the NIAID hepatitis B vaccine in such a setting.

"New" Antigen-antibody Systems Associated with HBV Infection - The possible etiological association between HBV infection and subsequent development of hepatic cell carcinoma (hepatoma) suggests the possibility that "neoantigens" may be present in HBV-infected cells or in hepatoma cells that contain the HBV genome. Dr. Rodney Johnson has tested sera from patients with hepatic cell carcinoma from Japan and South Africa for antibody that reacts with a human hepatoma cell line that contains the HBV genome but does not react with a HBV negative hepatoma line. He has found at least one antigen-antibody system that defines a nuclear antigen in the HBV-containing cells.

Integration of the HBV Genome into Host Chromosomal DNA - In a collaborative study with Dr. Gerin and Dr. Ding-Shinn Chen, we have sought evidence of integration of the HBV genome into chromosomal DNA from liver tissue of Asian patients with hepatoma. Such integration has been found at multiple and different sites within the high-molecular weight DNA extracted from hepatoma tissues. Surprisingly, integration was also found in non-tumor tissue obtained elsewhere in the livers of these patients with chronic HBV infection and hepatoma. Thus, integration itself does not appear to be synonymous with transformation although it may be a necessary prerequisite to transformation.

Non-A, Non-B Hepatitis Agents

Delta Antigen- In 1977, Dr. Rizzetto discovered a new antigen in patients with chronic type B hepatitis. Its distinctness from hepatitis B core antigen was demonstrated. Extensive studies with delta antigen were carried out by Dr. Rizzetto as a Fogarty Visiting Scientist in collaborative studies with Dr. Gerin's laboratory and the Hepatitis Viruses Section. Delta was found to be associated with a transmissible agent that required HBV synthesis for its own synthesis and that suppressed the synthesis of HBV markers. Thus, it had the characteristics of a defective interfering agent. Electron micrographic changes similar to those seen during acute non-A, non-B hepatitis infection have been detected in delta-infected chimpanzees, suggesting that delta may share some characteristics in common with other non-A, non-B agents. Delta appears to be a unique transmissible agent with characteristics unlike those of any previously described self-replicating agent.

"Classical" Non-A, Non-B Agents - Although satisfactory serologic tests have not yet been developed for non-A, non-B agents, Dr. Yohko Shimizu, while a Fogarty Fellow, did detect unique electronmicrographic changes in hepatocytes of chimpanzees experimentally infected with non-A, non-B agents. Two types of changes were observed, cytoplasmic changes associated with fusion of membranes of the endoplasmic reticulum into peculiar cylindrical structures and nuclear changes characterized by pycnosis, heterogeneous staining and, in a small proportion of cells, clusters of particles.

Dr. Stephen Feinstone has continued studies on the transmission of non-A, non-B agents to primates and has completed the infectivity titration of two strains, one that produces cytoplasmic changes and one that produces predominantly nuclear changes. The former has an infectivity titer of only approximately ten chimpanzee infective doses (CID) per ml; the latter has an infectivity titer of approx. $\geq 10^6$ CID per ml. Such titered virus suspensions will now be used to begin the characterization of these agents.

In addition, Dr. Feinstone has successfully transmitted non-A, non-B agents of both types to marmosets. The nuclear changes were reproduced in marmosets, but the cytoplasmic changes have not been seen to date.

Epidemic Non-A, Non-B Hepatitis - In collaborative studies with Dr. Khorshed Pavri, we have found that two outbreaks of epidemic water-borne hepatitis and one cluster of cases of endemic hepatitis in India could not be serologically related to infection with HAV or HBV. Thus, these cases, which resembled type A hepatitis in many respects, appeared to be caused by non-A, non-B agents. Attempts to transmit them to chimpanzees and marmosets are currently in progress.

Histopathology of Viral Hepatitis in Chimpanzees - In collaboration with Dr. Hans Popper, we have examined the similarities and dissimilarities of histologic changes occurring in the liver of chimpanzees experimentally infected with HAV, HBV or non-A, non-B agents. Subtle but distinct differences in histopathologic lesions caused by the three viruses were detected.

Attempts to Prepare Cell Substrates Suitable for Isolation of Hepatitis Viruses - Of all the hepatitis viruses, only HAV has been successfully isolated and serially propagated in cell culture. There is, therefore, a need for cell systems in which the hepatitis viruses can be readily isolated. Human and chimpanzee hepatocytes are, of course, susceptible to in vivo infection with all of the agents, but in vitro cultivation of primary hepatocytes is very difficult. A potentially useful approach is the development of hepatocyte hybridomas derived from fusion of differentiated hepatocytes with hepatoma cells that are immortal in culture. Dr. Daemer is attempting to produce human hepatoma-chimpanzee hepatocyte hybridomas that will retain from the hepatocyte parent the receptor sites and metabolic pathways necessary for systhesis of the hepatitis viruses and from the hepatoma the ability to grow continuously in culture.

Mycoplasma Research

Efforts to assess the significance of pathogenic spiroplasmas have centered on the development of appropriate animal models to explore host response and tissue localization of the agent. Experimental infection of suckling rats with the SMCA spiroplasma exhibits a number of features similar to those of certain "slow virus" diseases of man. For example, the organism grows to high titer throughout the brain producing variable spongiform changes without a clearly defined inflammatory response. Persistence of infection is also characteristic of the SMCA spiroplasma. When these tissues are examined by electron microscopy, helical organisms (or other microbial forms for that matter) cannot be visualized. Further studies are in progress to attempt localization of the spiroplasmas in brain tissue by specific immunological staining techniques. Studies on spiroplasmas and the development of appropriate culture media (SP-4) for the growth of the SMCA organism has resulted in some important contributions to our understanding of the mycoplasma flora of man. Using the SP-4 medium formulation, a newly discovered mycoplasma has been recovered from patients with non-gonococcal urethritis. The organism has a number of unique features, including a specialized terminal structure. This mycoplasma adheres to tissue culture cells by way of its tip and subsequently produces cytopathogenic effects in cell culture. Efforts to assess the frequency of infection by this organism and to determine its role in sexually transmitted disease are now underway.

Honors and Awards

Robert M. Chanock

Director, International Reference Laboratory for Respiratory Viruses Other Than Influenza, World Health Organization, 1973-present
Director, International Reference Laboratory for Mycoplasmas, World Health Organization, 1973-present
Associate Editor, American Journal of Epidemiology
Member, Advisory Board, Archives of Virology
Elected Foreign Member, Royal Danish Academy of Sciences and Letters, 1980
Reappointed member, World Health Organization Expert Advisory Panel on Virus Diseases, September, 1980-85
Section Chairman and invited participant, Symposium on Molecular Approaches to Viral Diseases in honor of Dr. Horace Hodes, Mt. Sinai School of Medicine, New York, June 1981
Recipient, Joseph E. Smadel Medal, and presented lecture at the annual meeting of the Infectious Diseases Society of America, September, 1980
Awarded the Robert Koch Prize from the Robert Koch Foundation, Bonn, Germany, December, 1981
Invited participant, Symposium honoring Prof. Frank Fenner, The Control of Viral Diseases in Southern Asia, Western Pacific and Northern Australia, sponsored by the Australian Academy of Sciences, February, 1982

Albert Z. Kapikian

Invited to attend and make 2 presentation (one as lecturer and one as discussant) at 3rd Nobel Conference on "Acute Enteric Infections in Children: New Prospects for Treatment and Prevention" in Stockholm (Saltsjobaden), Sweden, September 22-26, 1980. Presentation as lecturer on "New Developments in Viral Gasttoenteritis" on September 22, 1980. Presentation as discussant on "Other viruses-- practical considerations" on September 23, 1980.
Co-program chairman (with Dr. S. Matsumoto) of Panel on Viral Diseases, Working Conference on Rabies, Arboviruses Including Dengue, Korean Hemmorrhagic Fever and Viral Gastroenteritis. (Dr. Kapikian is panel member of the United States Panel on Viral Diseases of the U.S.-Japan Cooperative Medical Science Program.
Co-chairman (with Dr. T. Konno) of session on Viral Gastroenteritis On November 18, 1980 at Panel on Viral Diseases Working Conference on Rabies, Arboviruses Including Dengue, Korean Hemorrhagic Fever and Viral Gastroenteritis sponsored by the Japan-United States Cooperative Medical Science Program, Oiso, Japan.
Presentation on "Recent Advances in the Study of Rotavirus Serotypes" on November 18, 1980 at Panel on Viral Diseases Working Conference on Rabies, Arboviruses Including Dengue, Korean Hemorrhagic Fever and Viral Gastroenteritis sponsored by the Japan-United States Cooperative Medical Science Program. Oiso, Japan
Invited to attend a meeting of the Sub-committee on Rotavirus Reagents of the World Health Organization in Geneva, Switzerland, January 12, 1981. Dr. Kapikian is a member of this sub-group on rotavirus reagents.

Invited to participate in the Second Meeting of the Steering Committee of the Scientific Working Group on Viral Diarrheas of the World Health Organization in Geneva, Switzerland, January 14, 1981. Dr. Kapikian is a member of the steering committee and is listed as a member of the Scientific Working Group.

Invited to make brief presentation on viral gastroenteritis to Dr. Edward N. Brandt, Jr., Assistant Secretary for Health, Department of Health and Human Services on his visit with Dr. Frederickson et al. to Building 7, on March 9, 1981.

Invited to make presentation on "Parvoviruses" in the session on Infections caused by enteric viruses on June 4, 1981 at the Symposium on "Molecular Approaches to Virus Diseases" in honor of Dr. Horace Hodes. Distinguished Service Professor at Mount Sinai School of Medicine on June 4, 1981 at the Mount Sinai Medical Center, New York. Also invited to participate in a panel discussion of Infections Caused by Enteric Viruses at this Symposium, June 4, 1981.

Declined invitation to attend and make presentation on Viral Enteric Diseases on November 10, 1981 at the symposium "Immunobiology of Infectious Diseases" sponsored by the Sociedad Mexicana de Immunologis and the International Union of Immunological Societies, November 8-13, 1981, Oaxtepec, Mexico.

Invited to make presentation on "Prospects for development of a vaccine against rotavirus infection" on September 10, 1981 at a Symposium on Recent Advances in Enteric Infections, September 8-11, 1981 in Brugge, Belgium.

Robert H. Purcell

Invited speaker, Texas Society of Infectious Diseases, New Orleans, La., September, 1980.

Invited participant, International Workshop on Infectious Hepatitis, Athens, Greece, November, 1980

Invited speaker, Symposium on Viral Hepatitis, U.S.-Japan Bilateral Science Agreement, Hakone, Japan, November, 1980

Invited speaker, Chicago Gastroenterological Society, Institute of Medicine of Chicago, Combined Meeting, Chicago, December, 1980

Invited speaker, Pan American Health Organization, Washington, D.C. January, 1981

Invited speaker, Johns Hopkins School of Public Health, February, 1981

Invited particpant and member, organizing committee, International Symposium on Viral Hepatitis, New York City, March, 1981

Invited participant, Post-graduate training course" "Hepatitis Update", New York City, April, 1981

Invited speaker, American Academy of Pediatrics, Washington, D.C. April, 1981

Invited speaker, 40th Anniversary Symposium, Irwin Memorial Blood Center, June, 1981

Ching-Juh Lai

Recipient, Phoebe Weinstein Award for excellence in research on Negative Strand RNA Viruses, 1981
Invited participant, ICN-UCLA Symposium on Genetic Variation of Influenza Viruses, Salt Lake City, Utah, March, 1981
Invited participant, Gordon Research Conferences on Animal Cells and Viruses, Tilton, New Hampshire, June, 1981
Invited participant, V International Congress of Virology, Strasbourg, France, August, 1981

Joseph G. Tully

Past-Chairman and Member, Board of Directors, International Organization for Mycoplasmology (IOM)
Past-Chairman and Member of Board, IOM International Research Program on Comparative Mycoplasmology (formerly WHO/FAO Program)
Chairman, Subcommittee on Taxonomy of Mycoplasmas, American Society for Microbiology
Co-Director, Workshop of Basic Biology of Mycoplasmas, W. Alton Jones Cell Science Center, Lake Placid, N.Y. August 17-21, 1981.
Member, International Subcommittee on the Taxonomy of Mollicutes, International Commission on Systematic Bacteriology.
Member, Advisory Committee, Bergey's Manual of Systematic Bacteriology, 9th Edition.
Invited speaker, Department of Bacteriology, Cornell University, Ithaca, N.Y. October 14-15, 1980.
Invited discussant, Workshop on murine mycoplasma infections. Animal Resources Branch, NIH, Frederick Cancer Research Center, December 10, 1980.
Honorary Doctoral Degree, University of Bordeaux II, Bordeaux, France, November 17, 1980.
Invited speaker, International Conference on Pathogenicity of Mycoplasmas, Hebrew University and Kuvin Center for Infectious and Tropical Diseases, Jerusalem, Israel, May 19-21, 1981.
Invited speaker, Mycoplasma symposium. Annual meeting of the American Veterinary Medical Association, St. Louis, Mo., July 20-22, 1981.

Richard G. Wyatt

Consultant on U.S.-Egypt PL480 Project "Etiology of Viral and Nonviral Diarrheas(03-440-C), 1980--
Invited speaker, Nobel Conference 3: Acute Enteric Infections in Children--New Prospects for Treatment and Prevention. Stockholm, September 22-26, 1980. Presented talk entitled "Prospects for Immunization Against Rotaviral Disease."

Anthony R. Kalica

Invited Speaker, Working Conference on Rabies, Arborviruses including Dengue, Korean Hemorrhagic Fever and Viral Gastroenteritis - Japan-United States Cooperative Medical Science Program, Oiso, Japan, November, 1980.

Harry B. Greenberg

Invited speaker, U.S.-Japan Cooperative Medical Science Program, Viral Disease Panel, 1980

Invited speaker, V International Congress of Virology, Strasbourg, France, August, 1981

Michael M. Sveda

Invited participant, ICN-UCLA Symposium on Genetic Variation of Influenza Viruses, Salt Lake City, Utah, March, 1981

Brian R. Murphy

Consultant on Influenza Vaccines, World Health Organization, November, 1980

Invited speaker, ICN-UCLA Conference on the Genetic Variation of Influenza Viruses, Salt Lake City, Utah, March, 1981

Jorge Flores

First Prize, National Prize of Medical Sciences, 1980, awarded by the National Council for Scientific Investigations, Venezuela

Invited participant, poster session, V International Congress of Virology, Strasbourg, France, August, 1981

Judith G. Massicot

Student Research Presentations, First Prize, American Society for Microbiology, Washington, D.C. Chapter, 1981

Stephen M. Feinstone

Elected member, American Association for the Study of Liver Diseases, 1981.

Invited participant, International Workshop on Hepatitis A Virus Infection, Athens, Greece, November 1980.

Delivered the "Stern WinBlad Lecture", University of Lund, Malmo, Sweden, November, 1980.

Invited participant, International Symposium on Viral Hepatitis, New York, March, 1981.

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TITLE OF PROJECT (80 characters or less)
THE ETIOLOGY OF ACUTE INFECTIOUS NONBACTERIAL GASTROENTERITIS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are (1) to search for viruses which play an etiological role in the syndrome of acute infectious non-bacterial gastroenteritis of infants, children, and adults; (2) to cultivate (in vitro) the viral agents of acute infectious non-bacterial gastroenteritis; (3) to study the biophysical, biochemical, genetic, immunological and epidemiological characteristics of such agents; (4) to reproduce the syndrome experimentally for the purpose of a) studying the pathophysiological and immunological responses of the host, and b) assaying the infectivity of viruses such as human rotaviruses and the Norwalk and Norwalk-like agents; (5) to develop effective immunoprophylaxis for the viruses of acute, infectious, non-bacterial gastroenteritis; (6) to develop efficient and sensitive assays for a) detection of the viral and bacterial agents associated with gastroenteritis and b) antibodies to these pathogens.

Cooperating Units

Children's Hosital National Medical Center, Washington, D.C.; Johns Hopkins University, Balto., Md.; Center for Disease Control, Atlanta, Ga.; Meloy Labs., Rockville, Md., Plum Island Animald Disease Center, USDA, New York; University of Minnesota School of Medicine, University of Mass.; BOB, FDA; NINCDS; Veterinary Resources Branch, DRS, Dept. Veterinary Science, Ohio Agricultural Research and Development Center, Wooster, Ohio; Center for Vaccine Development, University of Maryland, Baltimore, Md.; University of Rochester, New York, State Health Department, Berkeley, Ca.; Institute of Biomedical Investigations, Mexico City, Mexico

Major Findings

Background - Important advances have been made in elucidating the etiological agents of acute infectious nonbacterial gastroenteritis, a syndrome that affects a broad segment of the population. It appears that viral gastroenteritis consists of at least two entities with distinct epidemiological characteristics. One, designated epidemic viral gastroenteritis, tends to occur in family or community-wide oubtreaks affecting adults and school-aged children, family contacts and probably young children as well. The illness is usually self-limited and characteristically lasts 24-48 hours. In 1972, we demonstrated by immune electron microscopy (IEM) that a 27 nm particle -- the Norwalk agent -- was associated with an outbreak of this form of gastroenteritis that occurred in Norwalk, Ohio. In addition, in further studies by our laboratory, particles that resemble the Norwalk agent morphologically have also been associated by IEM with two family outbreaks of gastroenteritis, on in Hawaii and the other in Montgomery County (MC), Md. The Norwalk and Hawaii agents appear to be distinct, whereas the Norwalk and MC agents appear to be related. An agent designated Ditchling or "W" has been associated with this form of gastroenteritis in studies in England. In addition, other "small round viruses" some with calicivirus-like morphology have been associated with outbreaks of gastroenteritis in various parts of the world. The other form of gastroenteritis, designated sporadic infantile gastroenteritis, represents a severe form of diarrhea affecting infants and young children, that often necessitates hospitalization and parenteral fluid therapy. It is evident now from studies in many parts of the world that the 70 nm human rotavirus is the major known etiological agent of sporadic infantile gastroenteritis. Before the discovery of this agent by Australian investigators in 1973, the etiology of the majority of cases of infantile gastroenteritis was unknown. Our gastroenteritis studies are focused on the rotaviruses as well as on the Norwalk group of viruses. The Norwalk group of agents has not been cultivated successfully in any in vitro system. While human rotaviruses are capable of initiating infection of cells in culture, characteristically the infective process is abortive and infectious virus is not produced. However, in spite of the failure to grow the Norwalk agent, our studies have elucidated the importance of the Norwalk virus as a cause of epidemic viral gastroenteritis in various epidemiologic settings. In addition a major breakthrough was described in a previous annual report - the successful serial propagation of human rotavirus type 2 in vitro. An additional major breakthrough described in last year's report marked the rescue of noncultivatable human rotaviruses by gene reassortment with cultivatable ts bovine rotavirus. This achievement has enabled us to study the antigenic relationships of various human rotavirus types. In addition, by examining reassortants we have been able to dissociate the neutralization and subgroup specifities of rotaviruses and have been able to discern the gene responsible for coding for the protein involved in each of these specificities for subgroup 2 human rotavirus and for the UK strain of bovine rotavirus. Also this past year marked our initial studies in volunteers with a human rotavirus strain which may hold promise as a potential candidate vaccine if it proves safe, antigenic, and able to induce protection. Highlights of research activities carried out by LID staff alone or in collaboration with others are described below.

Analysis of Antigenic Differences among Human and Animal Rotaviruses by Plaque Reduction Assay

The successful propagation of human rotavirus Wa and the rescue of noncultivatable human rotaviruses by genetic reassortment with bovine rotavirus, as described in previous annual reports has enabled us to pursue an analysis of the antigenic differences among various human rotaviruses and between human and animal rotaviruses by plaque reduction assay. Currently, a plaque reduction PR assay is being employed for measurement of antibodies to human rotavirus ("Wa"), bovine rotavirus ("UK" and "NCDV-Lincoln"), porcine rotavirus ("OSU"), simian rotaviruses ("SA-11," rhesus 1 and rhesus 2), as well as reassortant rotaviruses prepared by coinfection with a *ts*-bovine rotavirus and a series of non-cultivatable human rotaviruses. Plaquing of these rotaviruses is routinely carried out in MA104 cells. The PR assay as described in last year's report, was used to verify the identity of cultivatable human rotavirus (Wa) by comparing it with bovine, porcine, and simian rotaviruses. Human, bovine, and simian viruses were distinct when hyperimmune antisera were employed. However, when post-infection antiserum against Wa strain was used, a one-way relationship was observed between human and simian rotaviruses. Only post-infection antiserum was available for porcine rotavirus. Its use demonstrated porcine virus to be distinct from human and bovine viruses, but the porcine rotavirus was related in a one-way fashion to simian virus. The biological significance of these findings is not known at this time. It should be noted, however, as described in a previous annual report, that *in utero* infection of gnotobiotic calves with bovine rotavirus confers protection against subsequent challenge at birth with human subgroup 2 rotavirus, whereas an antigenic relationship between these viruses is not evident from cross-neutralization data.

Plaque reduction assay is now being used extensively to evaluate the antigenic relationships among reassortant viruses prepared from bovine rotavirus and non-cultivatable human rotavirus parents. One of the first human rotaviruses to be "rescued" *in vitro* by genetic reassortment was the DS-1 strain. This is a subgroup 1 virus that had been previously distinguished by ELISA and IAHA from the subgroup 2 Wa strain. When studied by neutralization in tissue culture, the Wa strain differed appreciably from the DS-1 reassortant (Table). A greater than twenty-fold difference was observed in both directions with the use of high-titered hyperimmune antisera.

A series of 20 additional noncultivatable human rotaviruses rescued by coinfection with cultivatable bovine rotavirus have been analyzed by PR assay. In the initial tests employing hyperimmune guinea pig antisera against Wa and DS-1, 14 of the strains resembled Wa and two resembled DS-1. Four did not react to high titer with either antiserum and are being characterized further. One of these viruses ("M") represents a distinct new serotype (Table). It is of added interest that this strain ("M") resembles simian rotavirus more closely in its neutralization characteristics than it resembles the Wa or DS-1 human rotavirus.

Table

Rotavirus	Reciprocal of antibody titer in serum from guinea pigs hyperimmunized with indicated virus		
	Wa	DS-1	M
Wa cultivatable mutant	8192	80	3290
DS-1 reassortant	80	31,273	80
M reassortant	80	80	81,920

Finally, it should be noted that the PR assay was used in the study that led to assignment of the neutralization protein to RNA segment number 9 as described later in this report.

Serologic response of infants and young children to rotavirus as studied by plaque reduction assay

The serologic response of infants and young children to primary rotavirus infection was studied by PR assay in order to evaluate the epidemiologic significance of the apparent antigenic differences described above. Sera from 10 patients, five who were infected with a subgroup 1 virus and five who were infected with a subgroup 2 virus, were studied by PR using the Wa and DS-1 reassortant strains of human rotavirus. Each of the 10 children developed a neutralizing antibody response to the Wa strain. In contrast, only five developed antibody to the DS-1 (reassortant) strain. Of the five, four had been infected with a subgroup 1 virus and one with a subgroup 2 virus. This degree of cross-reactivity was not anticipated, and its biological significance remains to be determined. Such cross-reactivity, however, does suggest that an effective human rotavirus vaccine may not require the inclusion of every serotype that exhibits significant antigenic variation when studied with hyperimmune antisera.

Further development of human rotavirus "Wa" strain for potential use as a vaccine.

As described in last year's report, we succeeded in developing a tissue culture adapted mutant of the Wa strain of human rotavirus. This mutant emerged during serial passage of this virus in gnotobiotic piglets. Triple plaque purification of this strain was carried out in primary African green monkey kidney cells by a technique in which (a) virus was first treated with trypsin prior to inoculation and (b) trypsin was added to the agarose overlay to facilitate plaque formation. A viral suspension (designated HRV-5) at the 16th passage level was then prepared and evaluated for safety prior to initiation of studies in adult volunteers. The ultimate objective is to determine if the Wa mutant is attenuated in the human host. If it is attenuated, we intend to evaluate its suitability for use as a vaccine.

This human rotavirus suspension has successfully undergone the appropriate safety tests to certify its freedom from testable adventitious agents. In addition to the routine safety tests in rabbits, guinea pigs, adult mice and suckling mice, the Wa strain (LotHRV-5) has been administered to newborn rhesus monkeys, gnotobiotic calves, and gnotobiotic piglets. The piglets which received wild-type Wa in the earlier studies that led to the emergence of the cultivatable Wa mutant, developed slight-to-moderate diarrhea during some of the passages. This clinical response was variable and unpredictable. Four piglets which received lot HRV-5 similarly developed mild diarrhea. While this does not suggest attenuation, it suggests that the virus did not become more virulent on passage in cell culture.

Three newborn colostrum-deprived rhesus monkeys were given wild-type Wa strain. None of the animals developed diarrhea, but each shed rotavirus in their feces; shedding was maximal 2 to 3 days after inoculation. Four monkeys which received HRV-5 also did not develop diarrhea. However, only two shed virus and maximal shedding occurred 4 to 5 days after inoculation. These observations suggested that HRV-5 grew less well in monkeys and might thus be attenuated.

A single calf received wild-type human rotavirus Wa. It subsequently developed diarrhea and shed virus for the first 4 days after inoculation. Three of five animals given the HRV-5 preparation developed diarrhea. These findings do not suggest attenuation of the HRV-5 preparation, but also they do not indicate increased virulence.

This HRV-5 preparation has now been administered orally to 12 adult volunteers. The first 2 volunteers had a high level of serum antibody to rotavirus, and did not develop illness. Virus was not detected in their stools, and their serum antibody titer did not increase significantly as measured by CF, IAHA, and PR assay. Since the first 2 volunteers did not develop illness, 10 volunteers with a low level of serum antibody to rotavirus were given the same preparation of virus (HRV-5). None became ill, and virus was not detected in the stools of the 10 volunteers. One volunteer had a single diarrheal stool 22 hours after receiving the virus, but virus was not detected in the stool, and the volunteer had no other signs or symptoms of illness. We could not conclude that this abnormal stool was associated with the viral inoculum. No untoward or unusual clinical reactions have been observed in the 12 volunteers who received the "Wa" human rotavirus thus far. Three of the second group of 10 volunteers, however, had mildly but distinctly elevated SGOT and SGPT values 10 days after receiving the virus, and the significance of this is being evaluated. Challenge of these volunteers who did not develop illness after HRV-5 administration, with the wild-type human rotavirus D strain (antigenically similar to Wa strain) is planned if the issue of elevated transaminases can be resolved completely. If the HRV-5 preparation of "Wa" strain is shown to be safe and antigenic, and to induce immunity it will be evaluated in increasingly younger age groups.

Antigenic Characterization of Human and Animal Rotaviruses by Immune Adherence Hemagglutination Assay (IAHA): Evidence for Distinctness of IAHA and Neutralization Antigens

Despite the fastidious nature of most human and animal rotaviruses, certain aspects of the antigenic properties of these agents have been elucidated. Initially, rotaviruses were found to share one or more common antigens by various techniques, including CF, FA, gel diffusion, and IEM. This property made it possible to use cultivatable animal rotaviruses as substitute antigens for detection of serologic evidence of infection with the fastidious noncultivatable human rotaviruses. Later, more specific antigens were detected by neutralization of abortive foci of infection in tissue culture, hemagglutination-inhibition, and ELISA blocking and binding techniques which demonstrated that human rotaviruses differed antigenically from animal rotaviruses and that animal rotaviruses differed from each other as well. Finally, using selective hyperimmune animal antisera prepared against complete virions of two strains of human rotavirus, i.e., virus that possessed a double capsid, it was possible to define by CF, IEM, and ELISA two antigenically distinct human rotavirus groups, designated serotype 1 and serotype 2. The existence of at least two human rotavirus serotypes was demonstrated by a fluorescent focus neutralization assay employing human convalescent sera or animal hyperimmune rotavirus antisera and was also suggested by a binding ELISA employing hyperimmune rotavirus antisera.

It had been assumed by us and others from different lines of evidence that the distinct human rotavirus serotypes detected by these methods would be identical to serotypes defined by conventional cell culture neutralization assay, once these fastidious noncultivatable human rotaviruses were adapted to tissue culture. Moreover, animal rotaviruses have been considered to be outside the realm of the human rotavirus typing schema since they were distinct antigenically from human rotaviruses by various techniques. As a matter of fact, in the ELISA previously employed in our laboratory for differentiating rotavirus type 1 and 2, the type specific antisera prepared in our laboratory were adsorbed with calf rotavirus antigen to remove common antibody in order to make the antisera more type specific. In addition, a typing test was not considered valid unless the type 1 and type 2 antisera reacted equally or almost equally with the bovine rotavirus antigen.

We have developed an IAHA for typing not only human but also certain animal rotaviruses employing antiserum obtained from a gnotobiotic calf infected with a type 1 or type 2 rotavirus. This was mentioned briefly in last year's annual report. These infection antisera were selected as they clearly differentiated human rotavirus type 1 and type 2 by IAHA when tested as endpoint serum titrations against a fixed amount of antigen or as endpoint antigen titrations against a fixed amount of sera. We examined the relationship of various animal rotaviruses to human rotavirus type 1 and 2. To our surprise, calf rotavirus strains NCDV and UK, the "O" agent and the simian rotavirus #1 and #2 were found to be clearly related to human rotavirus type 1. EDIM virus was not found to be related to either human rotavirus type 1 or type 2, whereas porcine rotavirus and simian rotavirus SA-11 reacted with antisera of 1 or 2 of the animal rotaviruses which exhibited type 1 specificity. The absence of reactivity of the animal rotaviruses with the human rotavirus type 2 antiserum was striking. The relationships noted above were essentially confirmed by the modified ELISA developed during this study; moreover, SA-11 was found to be closely related to human rotavirus type 1 in the ELISAs and porcine rotavirus was also related to human rotavirus type 1 by modified ELISA. The sharing of IAHA and/or ELISA specificity by the bovine, porcine, and simian (SA-11) rotaviruses was indeed an unexpected finding since each of these animal rotaviruses are distinguishable from each other by various neutralization assays. Thus, it became apparent that the neutralization and IAHA or ELISA specificities were clearly distinct properties.

As mentioned briefly in last years report, further evidence for the dissociation of the neutralization and IAHA antigenic specificities was obtained when we used IAHA to characterize viruses produced by genetic reassortment of noncultivatable human rotavirus "Wa" (IAHA type 2) and cultivatable UK bovine rotavirus ts mutant (IAHA type 1). The derivation of the reassortants is described in another section.

Four reassortants were studied in some detail. Each of these viruses was a reassortant since each virus derived at least 2 RNA segments from its human rotavirus parent and at least 6 RNA segments from its bovine rotavirus parent as determined by polyacrylamide gel electrophoresis. Reassortant 14-2 was specifically neutralized by human rotavirus "Wa" antiserum and was IAHA type 2, whereas reassortant 9-2 which was also specifically neutralized by human "Wa" rotavirus antiserum was IAHA type 1. Thus, the neutralization and IAHA antigenic specificities were clearly dissociable since reassortant 9-2 exhibited the IAHA reactivity of its bovine rotavirus parent and was neutralized specifically by antiserum directed against its human rotavirus parent. In addition, we also characterized by IAHA two reassortants designated 17-1 and 1-1, which were derived from coinfection of cell cultures with noncultivatable human rotavirus "Wa" (IAHA type 2) and cultivatable UK bovine rotavirus ts mutant (IAHA type 1) without the use of selective antiserum. Reassortant 17-1 was neutralized specifically by bovine antiserum (NCDV) and was IAHA type 1, whereas reassortant 1-1 which was also neutralized specifically by bovine antiserum (NCDV) was found to be IAHA type 2. Thus once again the neutralization and IAHA antigenic specificities were clearly dissociated in reassortant 1-1 but in a reciprocal pattern to that observed for reassortant 9-2. These observations suggest that the proteins that confer neutralization and IAHA specificities segregate independently during genetic reassortment and are thus coded for by different genes. Specific assignment of the genes responsible for the neutralization and IAHA antigenic specificities is described in a separate section. It should be noted that the IAHA antigenic characterization of the reassortants was confirmed by modified ELISA.

The relative importance to immunity of the separate proteins responsible for IAHA and neutralization antigenic specificities may be helpful in mounting a strategy for developing an effective rotavirus vaccine. The assumption has been that immunity to the specific human rotavirus serotype (as heretofore determined by specific CF, ELISA and now IAHA) was the factor which mediated resistance to illness. Although we have been able to type "core" rotavirus particles devoid of the outer capsid by IAHA, it is possible that the IAHA protein is exposed on the surface of the particle along with the neutralizing protein (as recently described by Hayes et al. for reoviruses) and thus might be related (directly or indirectly) to protection. Further studies are needed to assess the biological significance of antibody to the IAHA antigen.

Oral Administration of Rotavirus Strain "D" (Subgroup 2), to Volunteers: Induction of Illness and Correlates of Resistance

Although the epidemiology, natural history, and virologic aspects of human rotaviruses have been studied intensively, knowledge of the factors influencing susceptibility to rotavirus illness remains scanty. The need for such information is essential if successful methods of immunoprophylaxis are to be developed. From a series of six volunteer studies we gained some insight into the role of some of these factors. The first study was carried out at the Clinical Center, NIH and the remainder at the University of Maryland Center for Vaccine Development. Aspects of this study have been reported in previous annual reports. However neutralization tests on volunteers' sera and jejunal fluids employing the Wa strain and the DS-1 reassortant antigens have now been carried out and add important new information.

The inoculum was derived from a rotavirus-rich stool obtained from a 13-month-old child (D) hospitalized with a diarrheal illness. Five of 18 volunteers challenged orally with a 2% filtrate of the "D" strain (subgroup 2), shed rotavirus in their stools beginning on the second, third or fourth day after inoculation. Four of the 5 who shed rotavirus developed a diarrheal illness 2-4 days after inoculation. Analysis of the relationship between the development of illness and the absence of pre-inoculation serum antibody to human rotavirus (as measured by CF and IF) or calf rotavirus (as measured by neutralization) revealed a significant association only for the absence of IF antibody to human rotavirus.

As mentioned above, we have recently completed the testing of sera and jejunal fluids for rotavirus neutralization activity as determined by plaque reduction of a DS-1 reassortant (a subgroup 1 virus) and the cell culture adapted "Wa" virus (a subgroup 2 virus). The latter has been shown in the plaque neutralization assay cited earlier in this report to be similar if not identical to a "D" strain reassortant and distinct from the DS-1 reassortant. The relationship of the presence or absence of serum neutralizing antibody to the DS-1 reassortant or "Wa" virus and the development of illness following challenge with "D" strain was analyzed. An association was not observed since most of the volunteers possessed serum antibody to both the DS-1 reassortant and the "Wa" strain. However, the geometric mean serum neutralizing antibody titer to the two viruses was lower in volunteers who developed illness than in those who did not. It was striking, however, that only the difference in geometric mean antibody titer for the DS-1 reassortant was statistically significant. Volunteers who shed rotavirus had a significantly lower geometric mean serum antibody titer to both viruses when compared to individuals who did not shed rotavirus. Pre-inoculation jejunal fluid neutralizing activity for the "Wa" virus or DS-1 reassortant was not significantly different in the ill or non-ill volunteers included in the analysis. Five of the volunteers, including the 4 who became ill, developed a significant increase in serum neutralizing antibody to both the DS-1 reassortant and the "Wa" virus. An increase in neutralizing activity to both the DS-1 reassortant and the "Wa" virus was also observed in tests with paired pre- and post-inoculation jejunal fluids of several volunteers. The occurrence of a neutralizing antibody response to these antigenically distinct viruses raises a question about the need to include both serotypes in a rotavirus vaccine.

Of interest, 7 volunteers developed a 4-fold or greater rise in IAHA antibody for both DS-1 (IAHA subgroup 1) and Wa (IAHA subgroup 2); included in this group were the 5 individuals who shed virus. A similar pattern was also observed by ELISA. In contrast, the gnotobiotic calves inoculated with subgroup 1 or 2 rotavirus developed a subgroup specific response by IAHA but not by ELISA.

Two volunteers who developed diarrheal illness and laboratory evidence of rotavirus infection following initial administration of the "D" strain were given the same inoculum 19 months later. Neither developed a diarrheal illness nor vomiting after second inoculation, although one volunteer developed mild abdominal cramping the first and second days after inoculation and headache, anorexia, weakness and mild nausea 4 days after challenge.

The ability to induce illness in adult volunteers is important not only for attempting to gain an understanding of the mechanism of rotaviral immunity, but it also provides a basis for identifying susceptible individuals for future studies of candidate rotavirus vaccines. With the identification of correlates of susceptibility to rotavirus challenge, it should be possible to evaluate attenuation and efficacy of a candidate vaccine in adult volunteers. In such a study, a candidate vaccine or placebo would be administered to such susceptible volunteers (following previous safety tests, including administration to antibody-positive volunteers). These individuals would subsequently be challenged with wild-type rotavirus after allowing an appropriate interval for development of "vaccine-induced"immunity.

Development of Temperature-Sensitive ts Mutants of Animal Rotaviruses

As described in last year's annual report, as part of our effort to investigate and modify the virulence of rotaviruses, 10 ts mutants of the UK strain of calf rotavirus were isolated after mutagenesis with 5 azacytidine and/or 5 fluorouracil. Each of the mutants had a shutoff temperature for plaque formation of 38°C or 39°C and each was relatively stable genetically. Analysis of the ten mutants (M1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 13) showed that they belonged to four separate, non-overlapping, recombination groups. Polyacrylamide gel analysis of the RNA of these mutants indicated that each exhibited the same pattern as the ts^+ wild-type UK parent. In addition, we have isolated 6 ts mutants of the SA-11 simian rotavirus which fall into 3 non-overlapping recombination groups.

Other ts mutants have also been produced using a rhesus rotavirus originally isolated by Dr. Stukor, Oshiro, Schmidt et al. This virus has the advantage of having been passaged only in primary cell culture and of being rather avirulent in its natural host. Eight separate ts mutants have been isolated. These appear to fall in 3 separate reassortant groups. Thus far these ts viruses have been tested in only a very preliminary fashion for their ability to reassort with non-cultivatable human rotaviruses. Finally, one ts isolate of the porcine rotavirus OSU has been recovered.

Use of ts Mutants to Rescue Non-Cultivatable Human Rotavirus by Gene Reassortment

As described in last year's report, except for the "Wa" strain of subgroup 2 human rotavirus, human rotaviruses have not been successfully propagated serially in vitro to high titer. In addition, most animal and avian rotaviruses are extremely fastidious and cannot be cultivated in vitro by current cell culture techniques. For these reasons we developed a strategy for converting fastidious rotaviruses into viruses capable of efficient growth in tissue culture as described in last year's report. We took advantage of the high frequency of genetic reassortment of rotaviruses during mixed infection, to rescue non-cultivatable human rotaviruses by reassorting them with cultivatable ts bovine rotavirus. Initially, two human isolates were rescued (Wa and DS-1). These were shown to be serologically distinct in several neutralization assays. We have subsequently extended our rescue efforts and attempted to rescue an additional 31 human isolates from around the world. Of these 20 have been successfully rescued, i.e., they have yielded cultivatable reassortant viruses that were serotypically different from their cultivatable UK parent. These human reassortants have fallen into at least three distinct serotypes indicating that there is diversity in the neutralizing protein of human rotavirus. The plaque reduction neutralization data of these 3 serotypes were described in detail in a preceding section.

STUDIES WITH ROTAVIRAL RNA

A. Genes of Human Rotaviruses which Code for Neutralization and Subgroup Antigens and Which Restrict Growth in Tissue Culture

As described in last year's report, temperature-sensitive (ts) mutants of a cultivatable bovine rotavirus (UK strain) were used to rescue non-cultivatable human rotavirus (Wa strain). The resulting viral reassortants grew to high titer in cell culture and were neutralized specifically by human rotavirus antiserum but not by bovine rotavirus antiserum. By altering the selective conditions, we also recovered reassortants which were neutralized by bovine rotavirus antiserum but not by human rotavirus antiserum. The various reassortants reacted specifically when tested by enzyme-linked immunosorbent assay (ELISA) and the immune adherence hemagglutination assay (IAHA) which distinguish at least two subgroups of rotaviruses.

Since the rotavirus genome is segmented, it was anticipated that the viral reassorants produced during mixed infection would exhibit a variety of phenotypes. Analysis of reassortants genotypes allowed us to determine which gene was always present in viruses that were neutralized specifically by human rotavirus antiserum or bovine rotavirus antiserum. In this manner we identified the gene whose product reacts with neutralizing antibodies. In a similar manner, the gene coding for the viral protein reactive in the IAHA and ELISA subgrouping reaction also was identified.

Sixteen rotaviral reassortants recovered from mixed infection with a ts mutant of bovine rotavirus ("UK" strain) and non-cultivatable or cultivatable human rotavirus ("Wa" strain) were genotyped by PAGE. Analysis of the reassortants showed that the 9th RNA gene segment regularly segregated with the neutralization specificity and the 6th RNA gene segment regularly segregated with the subgroup antigenic specificity detected by ELISA and IAHA. This indicates that the 9th RNA segment codes for the protein that induces and reacts with neutralizing antibodies while the 6th RNA segment codes for the subgroup antigen.

The neutralization protein may be the rotavirus counterpart of the outer capsid σ1 protein of reovirus that is present in approximately 24 copies in the virion (Smith, et al). Although a minor component of the outer capsid, the outer capsid σ1 protein of reovirus is responsible for attachment to receptors on host cells and erythrocytes. Furthermore, antibodies to the outer capsid σ1 protein neutralize reovirus infectivity.

The position of the 6th gene product within the virion is not known for certain but two observations suggest that it may be a major inner capsid protein. First, purified cores of rotavirus retain subgroup antigenic specificity in apparently undiminished amount. Second, it has been shown by other workers that the 6th RNA segment of the simian rotavirus, SA-11, codes for a 42,000 dalton protein that is the major inner capsid protein of the virion. Additional genotypic analysis of the Wa-UK reassortants disclosed that Wa gene 4 was the only human gene not present in any cultivatable reassortant. This provides evidence that the 4th gene product of Wa virus is responsible for its lack of cultivatability. Further gene coding analyses have been done on two additional human rotavirus strains -- DS-1 and W. The genotypic analysis was accomplished by both ethidium bromide co-electrophoresis PAGE gels and by a newly developed hybridization technique. The findings indicate that the DS-1 strain has its neutralization protein coded for by the 8th gene while the W strain is identical to Wa with the 9th gene coding for the neutralization protein. In all human reassortants studied thus far, gene 4 has not been rescued and thus this gene appears to be responsible for restriction of growth in tissue culture.

B. Differences in Electrophoretic Mobility of Rotavirus dsRNA as an Aid in Identifying Subgroups

Last year we reported that analysis of 12 human rotaviral strains suggested a correlation of RNA pattern with ELISA "serotype." The immune adherence hemagglutination assay (IAHA) was employed in addition to the ELISA for serological grouping and the correlation of RNA with serological results was extended and confirmed with the testing of 11 additional human rotaviral strains: four subgroup 1 (formerly serotype 1) and seven subgroup 2 (formerly serotype 2). The subgroup 1 viruses had an RNA pattern with slower migrating 10th and 11th segments, whereas the subgroup 2 viruses had more rapidly migrating 10th and 11th segments. These results now extend the number of strains which have been studied to 23 viruses recovered from four geographic areas: Washington, D.C.; Bangladesh; Mexcity City, Mexico; and Caracas, Venezuela.

The practical significance of the correlation between RNA pattern and antigenic subgroup is the convenience offered by RNA analysis, since the large differences in migration of the smallest RNA segments between the subgroup 1 and 2 viral RNAs are easily resolved. Furthermore, this method offers an alternative which does not depend upon a supply of serological reagents for subgrouping that are presently not readily available. A relatively simple procedure for characterization of viral RNAs should facilitate the subgrouping of rotaviruses.

C. Transcription and Hybridization of Rotavirus RNA

The genetic material of rotavirus consists of eleven segments of double-stranded RNA (dsRNA). During infection, single-stranded RNA (ssRNA) copies of one strand of each segment are synthesized. These (+strand) ssRNAs act as messages directing the synthesis of the viral proteins. The transcriptive activity of the virus resides in the core and can be assayed in vitro. Under optimal conditions, complete transcripts of the viral genes can be obtained in multiple copies. Inclusion of radioactively labelled nucleotides during the reaction results in transcripts of high specific activity that can be used as probes for the recognition of RNA sequences in the viral genome. We have been studying this in vitro transcription activity and employing such probes with the following results:

(1) Products of the Transcription Reaction - Several human and animal rotavirus strains were purified and employed for preparative synthesis of ^{32}P ssRNA. They include the human strains Wa and DS-1, the calf strains NCDV and UK, the simian strain SA-11 and the simian rhesus strains Rh1 and Rh2. In every case we were able to obtain ^{32}P ssRNA that could be evaluated for its ability to hybridize to genomic RNA. For this purpose, unlabelled dsRNA from each one of the strains was denatured and allowed to hybridize to the corresponding ^{32}P ssRNA. The product was subjected to S-1 nuclease treatment to eliminate non-annealed ssRNA and analyzed by polyacrylamide gel electrophoresis and autoradiography. The incorporation of radioactivity into 11 double-stranded RNA segments that exhibited the same electrophoretic mobility as viral double-stranded RNA established not only the integrity of the ^{32}P ssRNA but also indicated that each one of the segments had been faithfully transcribed. The ^{32}P ssRNA was also analyzed by itself in agarose gels in which all the segments were clearly visible and equally represented. The order of electrophoretic mobility of these ssRNA segments, however, may not correspond to the mobility of the dsRNA segments; to sort out this possibility, experiments are in progress to extract the individual bands of ^{32}P ssRNA from agarose gel and identify the corresponding dsRNA segment by hybridizing ssRNA to genomic RNA.

(2) Relatedness of Different Rotavirus Strains - We have studied the relatedness of the above-mentioned strains by cross-hybridization of ^{32}P ssRNA obtained from one strain to dsRNA from another strain. Complete homology between equivalent segments of different viruses is revealed by the formation of hybrid (labelled) segments of mobility identical to that of the dsRNA segment; segments of partial homology yield the formation of smaller bands, caused by S-1 nuclease digestion of nonhomologous areas. By these analyses, we have found that our two prototype human strains, Wa and DS-1, share very little, if any, homologous sequences (in agreement with their serological differences). On the other hand, the two calf strains, UK and NCDV exhibited complete homology of at least four of their segments, while the two rhesus rotavirus strains, Rh_1 and Rh_2, were found to be identical throughout their entire genome although they were different from SA-11, another simian rotavirus. A more detailed analysis of homologous sequences shared by different viruses awaits the extraction of individual segments of single-stranded RNA that will be done selectively when more information about particular coding functions are assigned to the relevant segments (neutralization antigen, host range, ability to grow in vitro, etc.).

(3) Genotyping of Rotavirus Reassortants - Complete genotyping of several of the rotavirus reassortants described previously has been achieved by taking advantage of the low level of homology between the strains employed. For genotyping the UK x Wa reassortants, we prepared labelled probes from each parent; the unlabelled dsRNA from the reassortants is individually hybridized to the two probes and the hybrids analyzed by electrophoresis and autoradiography. The parental origin of each segment of the reassortant is established by the probe to which it hybridizes. Similar studies have been conducted with the UK x DS-1 reassortants and, more recently, the newer rescued human viruses.

(4) Molecular Epidemiology of Rotavirus Infection - Several rotavirus preparations obtained in Venezuela have been studied by cross-hybridization of their genomes among themselves and with our human prototype probes to determine RNA segment homologies and overall relatedness. During a study of 9 rotavirus-containing fecal specimens obtained July, 1980 from children with diarrhea hospitalized in a rehydration ward at the Caracas Children's Hospital, we found 6 different hybridization patterns. Findings suggest not only a high degree of genetic variability, but also the possible occurrence of natural reassortment between human strains. These results were surprising in view of the fact that all the samples were obtained at the same time from children of approximately the same age and similar socioeconomic and geographic backgrounds. Another set of samples comprising 42 specimens from the same hospital obtained between January and March 1981 confirmed this initial observation. Hybridization of the genomic RNA from these viruses to probes obtained from our DS-1 and Wa prototypes revealed the simultaneous existence of at least two families: some of them have a high degree of homology with DS-1 and none with Wa, while the remaining strains hybridized at varying degrees of homology with Wa, but not at all with DS-1.

The particular relevance of these findings to the subject of vaccine development has prompted us to undertake a more detailed longitudinal study in this Venezuelan population. This study is being conducted in cooperation with Dr. Perez-Schael at the Central University of Venezuela.

(5) Attempts to Clone Rotavirus RNA - In addition to the multiple benefits of studying and employing the in vitro transcriptase activity for genotyping, hybridization, and translation, the single-stranded RNA produced could be subjected to molecular cloning for gene amplification and potential expression of some of the more relevant viral proteins. We are currently in the process of developing the strategy for this approach. In order to make the rotavirus ssRNA a template for reverse transcriptase, it has to be polyadenylated, a reaction which we have successfully accomplished.

Since double-stranded RNA can also be polyadenylated, we have been using both ssRNA and dsRNA in attempts to synthesize DNA by reverse transcription. The synthesis of dsDNA and its insertion into an adequate plasmid will allow us to clone sequences and, hopefully, complete genes from the virus and eventually have those genes expressed. The possibility of developing a subviral vaccine by cloning the genes coding for the neutralizing antigens is highly attractive.

Rotavirus Proteins

A. Labelling with S-35 methionine

Work has continued on the study of rotaviral polypeptides using SA-11, UK, NCDV and Wa strains. The thirteen polypeptides of SA-11 as described in last year's report were confirmed in further experiments. In addition to the seven structural polypeptides reported last year, three new SA-11 structural polypeptides were found. One of the 3 which had been found in cell lysates had been designated non-structural previously and two which were previously not detected were found in purified virions but not yet in cell lysates. This brought the total SA-11 polypeptides to 15, the original 13 found in cell lysates prepared from infected MA 104 cells and 10 found in purified SA-11 double encapsulated (HA-positive) particles. Two of the structural proteins, a 57,500 dalton outer capsid protein and a 40,500 dalton inner capsid protein, were not seen in infected cell cytoplasmic lysates. In summary, the 15 SA-11 polypeptides were distributed as follows: 5 are nonstructural, 5 are inner capsid and 5 are outer capsid proteins. Two of the cytoplasmic lysate proteins (50,500 dalton and 30,000 dalton nonstructural proteins) appeared to be early proteins in that they were more abundant at 4 hours post-infection than at 8 or 12 hours. In addition, a 38,000 dalton inner capsid, 26,000 dalton and 24,000 dalton outer capsid and 22,200 dalton nonstructural proteins appeared to be later proteins in that they were more abundant at 8 hours or later in the infection cycle. More precise pulse labelling is planned to further substantiate these findings.

Radioactively labelled purified SA-11 virions and core particles (with densities of 1.36 and 1.38 g/ml, respectively), when subjected to trypsin treatment (2 ug/ml or 10 ug/ml) and analyzed by PAGE, resulted in the disappearance of two outer capsid proteins (77,000 and 57,500 daltons) and one inner capsid protein (40,500 daltons). Three new proteins appeared (56,000, 53,500 and 41,500 daltons) regardless of the concentration of trypsin was used. Three additional proteins were also present in each preparation and their size was dependent on the concentration of trypsin used -- (2 ug/ml -- 56,000, 29,000 and 21,000; 10 ug/ml -- 54,000, 27,800 and 26,000). It is interesting that the 77,000 dalton-outer-capsid-4th gene product of SA-11 is trypsin-sensitive since data obtained with reassortant viruses indicates that the 4th gene may be associated with growth restriction as described previously in this report. Core particles were unaffected by trypsin at concentrations of 2 or 10 ug/ml.

Since last year's report one additional polypeptide was seen in cytoplasmic lysates from cells infected with Wa, UK and NCDV, bringing the total to 11, 12 and 10 respectively for each of these viruses. Seven Wa structural polypeptides have been identified with at least 4 of them located on the inner capsid and one on the outer capsid. Two were not assigned and require further examination. Six structural proteins have been identified for the UK calf viruses, 4 are inner and 2 are outer capsid proteins. Some progress has also been made in obtaining higher titered inocula for biochemical experiments with these viruses and work will continue in an attempt to identify all of their polypeptides.

B. Translation of Rotavirus Proteins

Unlabelled rotavirus mRNA synthesized in vitro is being employed to study the protein composition of human (Wa) calf (UK, NCDV) and simian (SA-11, Rh 2) rotaviruses. For this purpose, we are using the rabbit reticulocyte lysate in vitro translation system. The viral mRNA can "program" this system to synthesize labelled viral proteins which can be compared by polyacrylamide gel electrophoresis to labelled proteins synthesized by the virus during infection of cell cultures and to the proteins of complete labelled viruses. Side-by-side analysis of the protein patterns in the gels reveals the synthesis of the major viral proteins. Small differences in migration observed suggest post-translational modification of some of them; more relevant information that will allow us to make gene-coding assignments will be gained by translation of mRNA extracted from individual bands after its separation in agarose gels. In this regard, with several viruses we have already been able to individually translate the 3 to 4 fastest-moving segments of single-stranded RNA after agarose electrophoresis.

The combination of in vitro translation with immunoprecipitation will be useful in the characterization of the hybridomas which are being developed. Important information may also be gained by using the in vitro transcription-translation system in the study of proteins (and their serological reactivity) of non-cultivatable human viruses. In this regard, we have already obtained preliminary information on the human DS-1 strain.

C. Glycoproteins of Rotavirus

We have also been investigating the existence and location of glycoproteins in the rotavirus virion by incorporating radioactive sugar precursors into their glycosidic moieties. For labelling glycoproteins, the culture is subjected to a short period of glucose deprivation followed by addition of ^{14}C-glucosamine; the incubation is allowed to proceed and stopped at various times when the cells are lysed with SDS and the product is electrophoresed in polyacrylamide gels. Uninfected cells undergo the same treatment and serve as controls. The labelled bands are compared with preparations labelled with ^{35}S-methionine. Three proteins of approximate molecular weights of 77,000, 38,000, and 23,000 daltons are labelled when cells are infected with the human (Wa) rotavirus. They correspond with proteins of similar size that are labelled with ^{35}S-methionine. Although we have tried to label complete virus with ^{14}C-glucosamine, the specific activity of the product is quite low. An alternative way to determine the presence of glycoproteins in the virion has been to treat the ^{35}S-labelled complete virus with a protease-free oligosaccharidase and to look for changes in the mobility of the proteins. By using this approach we have confirmed that the 77,000 and 38,000 dalton weight proteins are indeed glycosylated; however, the small (23,000 daltons) protein is not seen in those preparation. A third way to study the glycosylation phenomenon has consisted in separating the proteins of unlabelled viral preparations by electrophoresis and transferring them onto nitrocellulose paper by electroblotting. The paper is then incubated with ^{125}I-Concanavalin-A and autoradiographed. By using this technique we have been able to detect the 38,000 daltons component; however the efficiency of the blotting seems to be low for large proteins.

Obviously more work is required in this area, especially in connection with the study of the function of the glycoproteins and their serological reactivity. We are planning to undertake such studies once we have fully identified these proteins.

Development of Hybridomas for Human Rotavirus

The project was initiated in order to utilize the inherent specificity of monoclonal antibodies for investigating the biologic role and structure of various rotavirus proteins. In addition, it was hoped that specific serologic reagents could be developed that would aid in the epidemiologic study of human rotavirus illness as well as in the preparation and testing of candidate rotavirus vaccine strains.

Standard mouse myeloma cell fusion techniques involving the use of NS1 cells, HAT selection media and terminal dilution cloning of candidate hybridomas were employed. Balb C mice were immunized with partially purified human rotavirus (Wa strain, subgroup 2) and with reassortant viruses 13-2 and 41-2 (776 x M11B), that are serotypically identical to human rotavirus, DS-1 strain (subgroup 1). Immunization schedules varied but always involved one inoculation of virus in complete Freund's adjuvant and one booster that included virus in incomplete Freund's adjuvant followed by one or more additional inoculations of rotavirus in an aqueous suspension. Antibody-producing clones were identified by solid phase RIA and/or indirect immunofluorescence. In both cases, an anti-mouse IgG detection system was employed.

A variable yield was obtained from different fusions. Thus far we have isolated 11 stable, independently selected, doubly cloned monoclones directed against the Wa strain human rotavirus. Characterization of the monoclones is incomplete; however, 4 appear to react exclusively or almost exclusively with human serotype Wa rotavirus. Two of these four monoclones neutralize virus as indicated by plaque reduction assay. An additional monoclone appears to react with the gene 6 subgroup protein. The remaining seven monoclones cross-react to a varying degree with various animal rotaviruses. None of the cross-reactive monoclones appear to have neutralizing activity. Two approaches are being taken to investigate which viral protein the individual monoclones are directed against. First, attempts are now underway to immunoprecipitate with the hybridoma monoclones labelled rotavirus proteins and identify the precipitated protein by polyacrylamide gel electrophoresis. Second, hybridomas are being tested for reactivity with a variety of rotavirus reassortants of defined genotype that possess a mixture of genes derived from their human and bovine rotavirus parents. In this manner it should be possible to determine the gene product that reacts with each non-cross-reactive hybridoma antibody.

Development of Chymotrypsin Sensitive Mutants of Bovine and Porcine Rotavirus to Use in Gene-Function Genetic Reassortment Studies

Choppin *et al.* have shown that "protease activation" mutants of Sendai virus can be selected and that these mutants can be used to investigate the biologic function of the Sendai fusion (Fo) glycoprotein. All rotaviruses are partially or completely dependent upon protease activation for efficient growth and plaque formation. The Wa strain of human rotavirus will produce plaques on MA104 cells in the presence of trypsin or elastase. Plaques do not develop, however, in the presence of chymotrypsin.

We have selected bovine (UK strain) mutants that plaque and grow efficiently in the presence of chymotrypsin. Reassortment experiments are planned in which MA104 cells will be coinfected with Wa virus and chymotrypsin-sensitive UK virus. Growth yield will be plaqued out with chymotrypsin in the overlay as well as bovine rotavirus antiserum (specific for gene 9 product). Genotypic analysis of plaques derived from this experiment should permit us to identify the gene coding for the protein that is modified by protease. This protein may play an important role in infectivity and tissue tropism.

Characterization of Feline and Canine Rotaviruses

Cloning by plaquing and preparation of standard suspension of both feline and canine rotaviruses are underway in order to further study physico-chemical and biological properties of both agents by a variety of tests including RNA analysis, IAHA, HA and neutralization. Genetic studies on the relationship between plaque size and virulence properties of feline and canine rotaviruses are in progress.

ROTAVIRUS ENVIRONMENTAL STUDIES

A. Rotavirus survival on environmental surfaces.

Studies will be initiated on the survival of NCDV on various environmental surfaces, including plastic (polystyrene, vinyl and polyethylene), glass, cotton, latex rubber, stainless steel and aluminum. In these experiments small surfaces (1-5 cm^2) are inoculated with a 10-100λ of high-titered virus ($\sim 10^7$-10^8pfu/ml) suspended in either tissue culture medium (Eagle's MEM) or stool suspension (20% in distilled water). The surfaces are incubated at room temperature under controlled relative humidity (RH) conditions, ranging from 30-95%. At various times post-contamination, the inoculum is recovered from the surface by resuspending in diluent and assayed for remaining virus infectivity by the plaque technique. From these experiments it should be possible to determine the kinetics of NCDV inactivation at various RHs as a function of time.

B. Detection of Rotaviruses in Edible Shellfish

Because of continued outbreaks of shellfish-borne gastroenteritis, the significance of rotaviruses as the etiologic agents of such outbreaks is of interest. In order to determine if rotaviruses are significant contaminants of shellfish, methods for rotavirus detection in suspect shellfish are needed. As an initial approach in the development of such methods, the methods that have previously been developed for detecting enteroviruses, reoviruses and adenoviruses will be employed in an attempt to recover two rotaviruses (NCDV and SA-11) from experimentally contaminated oysters and clams. In such methods viruses are eluted from contaminated shellfish homogenates using pH 7.5 glycine (.05 M) -NaCl (0.15 M). The viruses in the elution fluid are further concentrated by acid precipitation at pH 4.5. The precipitate is recovered by centrifugation, redissolved in a small volume of phosphate-buffer, and treated with a cationic polymer to remove components that are toxic to cell cultures. The treated material is then inoculated into cell cultures for assay of virus infectivity.

Role of Norwalk Virus in Acute Infectious non-bacterial Gastroenteritis

The development of a radioimmunoassay (RIA) for detection of Norwalk virus antigen and a radioimmunoassay blocking test (RIABL) test for detection of Norwalk antibody were described in a previous annual report. The availability of the RIABL has permitted continued epidemiological studies of Norwalk infection. As reported in last year's annual report, approximately 75 separate outbreaks of gastroenteritis had been studied serologically for Norwalk infection and about one-third appeared to be associated with Norwalk virus. Recently, Dr. Kaplan and colleagues have analyzed outbreaks of gastroenteritis which have been studied etiologically by the Centers for Disease Control, Atlanta, in collaboration with LID; the Norwalk serology was performed at LID. In their analysis 31 (42%) of 74 oubreaks of acute epidemic non-bacterial gastroenteritis between 1976-1980 were associated with Norwalk virus. The vehicle of transmission was known in 17 instances: 13 times it was water and 4 times food. The occurrence of symptoms by age were analyzed in five of the outbreaks and it was found that in children, vomiting occurred more frequently than diarrhea, whereas in adults diarrhea occurred more frequently than vomiting.

Longitudinal studies of infection with Norwalk virus (and rotavirus) in children on two Panamanian San Blas Island and in rural Bangladesh were studied in collaboration with Dr. Ryder et al. and Dr. Black et al. Serum antibodies to Norwalk virus (and rotavirus) were measured as part of a longitudinal study of infectious diseases and nutrition in two Panamanian San Blas Islands. During the nine-month study the incidence of diarrhea in children younger than five years of age (40% of the population was under five) was 32 per 100 children. The prevalence of antibody to Norwalk virus (and rotavirus) increased rapidly with age. The frequency of Norwalk seroresponse was 35% and was highest in children in their second and third years of life. Children without a Norwalk seroresponse had significantly fewer diarrheal episodes than children with a response. This study suggests that Norwalk virus infection was associated with mild gastroenteritis in children in Panama. A longitudinal study of diarrheal illness in rural Bangladesh also showed a rapid acquisition of Norwalk antibody with increasing age. During the one-year of study, the incidence of Norwalk antibody seroresponse was also highest in children in their second and third years of life. Excess occurrence of diarrheal illness was found in children who had a Norwalk virus seroresponse. These studies indicate that Norwalk virus and perhaps other serologically distinct 27 nm viruses are associated with a portion of diarrheal episodes in children in developing countries. Nearly all children tested satisfactorily by ELISA had serum antibodies to rotavirus at the beginning of the study. Children with low levels of rotavirus antibody had a higher risk of rotavirus diarrhea than children with a higher level of antibody and the titer of antibody increased with age. Subclinical rotavirus infections occurred frequently.

Other Agents

In previous annual reports we described studies with another 27 nm particle, the Marin County agent wich was clearly associated with an outbreak of gastroenteritis in a nursing home in California by Dr. Oshiro et al. This particle did not appear to be related serologically to the Norwalk, Hawaii or Snow Mountain agents. In last year's annual report we reported the administration of the Marin County agent to two chimpanzees, neither of which become ill or developed a seroresponse. In addition, we reported the administration of the Marin County agent to two volunteers, one of whom developed a mild gastroenteritis but neither of whom shed viral particles detectable by IEM. These studies were aimed at generating virus positive stools so that attempts could be made to develop a radioimmunoassay for this agent. This goal was pursued further by administration of a Marin County agent containing suspension to 15 additional volunteers; none developed definite illness. Selected stools from volunteers were also examined for virus particles and found to be negative.

We also administered the Snow Mountain agent to 7 volunteers in an attempt to generate virus positive stool material so that a radioimmunoassay might be developed. This agent has been associated with a waterborne outbreak of gastroenteritis in a camp in Colorado by Morens et al. In their studies a filtrate derived from this outbreak induced a gastroenteritic illness in volunteers. In our study, none of the seven volunteers developed a gastrointestinal illness. We have very recently learned that approximately four months after challenge, one of the volunteers has developed frank hepatitis (hepatitis B surface antigen negative, hepatitis A-antibody negative in a serum specimen) and another volunteer, although not clinically ill, is hepatitis B surface antigen positive in a serum specimen. These developments will be followed up carefully.

Crohn's Disease

Acute viral gastroenteritis has been suggested to be a precipitating factor in relapses of inflammatory bowel disease (IBD) activity. If this is the case, immunization against enteritis viruses, when and if available, could aid in the management of IBD patients. In collaboration with Drs. Gebhard, Henry, Sharp and Kaplan of the University of Minnesota an effort was made to determine whether viral gastroenteritis infection is an important potentiator or exacerbator of IBD activity. Seventy-seven (77) IBD patients were followed at regular intervals for 3-31 months (mean = 14 months). Patients (aged 11-64 years) included 57 with Crohn's disease and 20 with ulcerative colitis as well as 10 normal controls. At approximately three-month intervals and during exacerbations, activity of patients' disease was assessed using the Crohn's Disease Activity Index (CDAI), serum was obtained for viral serology and stool was obtained for detection of viral antigens. An exacerbation of disease was defined as a rise in CDAI >100 above baseline in conjunction with the clinical impression of worsened disease. Serum was tested for antibody to Norwalk agent by RIA and for antibody to rotavirus by complement fixation. Stool was tested for antigens of 3 predominant enteritis virus: Norwalk agent (RIA assay), rotavirus (ELISA assay), and adenoviruses (ELISA assay). During the study period, 65 exacerbations were observed. Viral antigen was not detected in any patient or control stools (266 samples), regardless of disease activity. Four adult Crohn's disease patients showed a significant four-fold rise in antibody titers associated with apparent increased disease activity (2 = Norwalk, 2 = rota). One adult Crohn's disease patient showed a fourfold titer rise to rotavirus without clinical change in activity of his disease. None of the 11 pediatric patients showed a change in antibody titer. These data suggest that acute viral enteritis does not play a substantial role in precipitating exacerbation of IBD.

Disease Due to Enterotoxigenic Escherichia coli (ETEC) in Bangladeshi Adults: Clinical Aspects and a Controlled Trial of Tetracycline

This study was carried out in collaboration with Drs. Merson, R.B. Sack, Islam, Saklayen, Huda, Huq, Zulich, and Yolken with the LID performing the serologic tests for rotavirus (CF)(published June, 1980, J. Infect Dis.). They studied 176 adult males presenting to the Cholera Research Laboratory Hospital in Dacca, Bangladesh (now the International Centre for Diarrhoeal Diseases Research, Bangladesh) from October to December, 1976 with a history of acute watery diarrhea, who appeared to have lost $\geq$ 5% of body weight. The admission stools were negative for *Vibrio* cholera by dark-field microscopy. A potential pathogen was isolated or detected in stools from 147 (86%) of the 176 patients. ETEC were isolated from 109 (62%) of the patients. It was of interest that 9 (5%) of the 176 patients had serologic evidence of rotavirus infection (8 of these also shed rotavirus in stool). However, in only 1 of the 9 was rotavirus the only pathogen detected. Eighty-eight of the 109 ETEC patients had only ETEC isolated from their stool and also reported not taking tetracycline before admission. These patients included 55 with heat-labile toxin (LT) and heat-stabile toxin (ST) producing, 30 with ST producing and 3 with LT producing ETEC strains. The clinical characteristics of disease associated with ETEC alone were determined in these 88 adult males admitted with moderate-to-severe dehydration. Persons infected with ETEC strains producing both LT and ST had more dehydration and acidosis, longer duration of illness, and greater stool volume than persons infected with strains producing only ST. Tetracycline therapy, evaluated in 63 cases, resulted in slightly earlier termination of illness in the patients with LT-ST strains, but had no effect on illness in the patients with ST strains. In both groups of patients tetracycline shortened the duration of excretion of organisms. Because of the limited effectiveness and the generally excellent response of ETEC diarrhea to rehydration therapy alone, it was concluded that tetracycline was not warranted for use in treatment of ETEC diarrhea in adults in this population.

Biweekly Prophactic Doxycycline for Travelers' Diarrhea

This study was carried out collaboratively with Drs. Santosham, R.B. Sack, Froehlich, Yolken, Javier, Medina, and F and I Ørskov, with the LID performing the Norwalk virus and rotavirus serologic tests on paired sera (RIA blocking and CF, respectively). A double-blind study to determine the efficacy of biweekly oral doxycycline in the prevention of travelers' diarrhea was conducted among 46 Peace Corps volunteers during their first six weeks in Honduras. The volunteers took either 100 mg of doxycycline per dose or a placebo for three weeks and were observed for an additional three weeks. There was no signficant difference in the number of persons with travelers' diarrhea in the two groups (eight of 24 in the doxycycline group and 10 of 22 in the placebo group) in the three weeks when the drug was taken. However, significantly fewer episodes ($P < 0.05$) of travelers' diarrhea occurred in the doxycycline group than in the placebo group at the end of the second, third and fourth weeks. Enterotoxigenic Escherichia coli (ETEC) was the most common pathogen identified. ETEC from 13 (62%) of 21 stool samples were resistant to doxycycline. Biweekly doxycycline was only marginally effective in preventing travelers' diarrhea and did not prevent diarrhea seconday to doxycycline-resistant ETEC. It was of interest that 4 (10%) of the Peace Corps volunteers had serologic evidence of recent infections with the Norwalk virus. Each of the four volunteers was symptomatic. Two of these volunteers also had ETEC isolated from their stools between the third and sixth weeks. None of the volunteers had serologic evidence of recent infection with rotavirus.

Relevance of Project to Missions of NIAID

Acute infectious nonbacterial gastroenteritis is a common infectious disease which affects a broad segment of the population and was the second most common disease observed in a 10 year family study in the United States. In addition, diarrheal diseases in general are a leading cause of mortality as well as morbidity in developing countries. (In the developing countries, rotaviruses are known to cause diarrhea but their relative role in the high mortality rate is not yet known.) Ultimate goals of prevention and therapy of acute infectious non-bacterial gastroenteritis of viral origin have been furthered in our current studies by (i) the detection and preliminary characterization of etiologic agents of this disease, (ii) the development of sensitive, efficient and rapid assay systems by which the epidemiologic importance of the disease can be determined, (iii) the in vitro cultivation of type 2 human rotavirus, (iv) development of a DS-1 (subgroup 1) human rotavirus reassortant that grows well in cell culture, (v) development of an efficient method to rescue other non-cultivatable human rotaviruses by gene reassortment, (vi) the ability to induce illness in human volunteers and experimental animals administered the human rotavirus, (vii) development of biophysical and serologic methods to differentiate the human rotavirus from other related agents and (viii) the elucidation of the epidemiology of the rotavirus and Norwalk viruses.

Proposed Course of Project

In future studies of the Epidemiology Section, major emphasis will be given to the efficient propagation of viral gastroenteritis agents in vitro, to the delineation of their overall importance over a sustained period in the etiology of gastroenteritis in various populations, to the immune mechanisms involved in host-defense, to the development of effective methods to prevent illness due to these agents, and to the continued search for other etiologic agents of acute infectious gastroenteritis.

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PI. Brian R. Murphy, M.D.	LID, NIAID	Medical Officer
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COOPERATING UNITS (if any)
Flow Laboratories (contract # N01 AI32521); NINCDS; Bureau of Biologist, FDA, National Cancer Institute, Immunology Branch, St. Jude Children's Hospital, Memphis, Tennessee; Cornell Univ. School of Veterinary Medicine, Ithaca, NY

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SUMMARY OF WORK (200 words or less - underline keywords)

A new ts donor virus has been constructed for possible use in immunoprophylaxis. This virus contains ts P3 and ts NP genes and is more stable than the ts-1[E] or ts-1A2 donor virus. Intrasegmental complementation was observed with mutants bearing their lesion in the P1 gene. This suggests that this polypeptide has at least two functional domains. Wild-type H1N1 NP gene was observed to suppress the ts phenotype of a ts P1 gene. Certain avian influenza A viruses were found to be attenuated for squirrel monkeys. Hemagglutinin-specific ELISA was used to characterize isotype-specific immunoglobulin response to primary and secondary infection with influenza virus. Local IgA ELISA antibody to HA correlated with resistance to infection with ca vaccine virus. Lymphocytes cytoxic to influenza A virus infected target cells were detected directly in peripheral blood of volunteers undergoing infection with influenza A virus. Lymphocytes producing IgA and IgG antibodies to hemagglutinin were detected in serum and blood of infected volunteers.

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Production of a New "ts-1[E] like" Donor Virus

A new "ts-1[E] like" virus was produced by mating a single lesion mutant possessing a ts defect on the P3 gene with another single lesion mutant bearing a ts NP gene. Recombinant viruses that contained ts P3 and NP lesions were isolated. The original ts-1[E] donor virus possessed ts mutations on the same two genes. The single lesion donors of the ts P3 and NP genes were more stable genetically than the double-lesion ts-1[E] master strain and were chosen to produce the new ts donor virus for this reason. Three recombinant viruses were isolated, clones 20, 53 and 55, which had the following properties: (i) ts P3 and ts NP genes (by genetic analysis); (ii) 37°C to 38°C shutoff temperature for plaque formation; (iii) 10,000 fold restriction of replication in hamster lung (approximately 100-fold more restricted than the master ts-1[E] virus); (iv) no significant restriction of growth in the nasal turbinates of hamsters; (v) genetic stability in hamsters in that all isolates obtained from the lungs (12 tested) and nasal turbinates (83 tested) were ts, and (vi) replication of the mutant at 37°C (during an in vitro stress test) yielded virus that was more temperature sensitive (38°C shutoff) than the progeny of ts-1A2 recombinants subjected to the same stress.

In order to further examine the genetic stability of this new reassortant virus, hamsters were infected either with the Udorn/72-clone 55 or the Udorn/72-ts-1A2 reassortant and then adminstered cyclophosphamide to inhibit the immune reaponse of the animal in order to prolong virus shedding. Normal hamsters stop shedding virus by day 5, whereas cyclophosphamide treated animals shed virus until day 9. Each isolate obtained from nasal turbinates on day 4, 7 or 9 was characterized for its efficiency of plaque formation at 34°, 36°, 37°, 38° and 39°C. Virus isolates from hamsters infected with clones 1A2 or 55 obtained on day 4 and 7 retained the 37°C shutoff temperature of the parent virus. On day 9, 11 of 13 isolates from hamsters infected with clone 55 had a 37°C shutoff (2 had a 38°C shutoff), whereas only 5 of 13 ts-1A2 isolates had a 37°C shutoff temperature (8 had a 38°C shutoff) ($p < .02$ Fisher Exact test). These results indicate that the clone 55 virus is more genetically stable than the ts-1A2 virus after prolonged replication in hamsters. This study is being repeated, and if confirmed, would suggest that reassortants derived from this new donor should be evaluated for safety, antigenicity and stability in man.

The clone 20 virus described above (ts mutations affecting the P3 and NP genes) was mated with a wild type equine influenza A virus (Heq1 Neq1) and 2 ts equine reassortants were isolated. These studies were carried out in collaboration with Dr. Leroy Coggins and Dr. James Gillespie at the University of Cornell School of Veterinary Medicine, Ithica, NY. The A/Cornell/64 ts recombinant clone 71 (Heq1 Neq1) had the following properties: (i) ts NP and ts P3 genes (by complementation analysis and genotyping), (ii) hemagglutinin and neuraminidase proteins of the equine parent, and (ii) growth and genetic stability characteristics in hamsters identical to the clone 20 ts parent. The other clone (clone 8) had only the ts NP gene.

The two equine ts reassortants were genotyped and the results confirmed the inheritance of the ts P3 and ts NP genes from the human parent in clone 71 and the ts P3 gene in clone 8. Furthermore, genotype analysis confirmed that these clones were reassortants and contained a mixture of human and equine genes. The ts equine reassortant clone 8 was satisfactorily attenuated and immunogenic in horses kept in isolation at the Veterinary College in Ithaca, New York. These equine ts reassortants have two properties that probably contribute to attenuation for horses: (1) the ts phenotype and (2) a mixed equine-human gene constellation that may be partially restrictive in horses. Evaluation of this vaccine candidate should be pursued in horses because this method of vaccine production may have relevance to the construction of influenza A donor viruses for use in human vaccines, i.e., ts mixed gene constellation human-animal or human-avian viruses. If the ts genes in such human-animal ts reassortant vaccine viruses revert, attenuation and lack of transmissibility might not be lost because a mixed gene constellation would not be expected to function efficiently in man.

Genetic Characterization of ts Reassortant Progeny

The A/Udorn/72-ts-1A2 donor virus (H3N2), with ts mutations on its P3 and P1 genes, was mated with the A/Hong Kong/77 (H1N1) wild-type virus in order to produce attenuated ts Hong Kong/77 reassortants. Two H1N1 ts progeny from this reassortment, clones ts-104 and ts-127, belonged to complementation/recombination (C/R) group 5 since they failed to complement the A/Hong Kong/68-ts-315 (H3N2) virus, the prototype of our C/R group 5 which contained a ts mutation on the P1 gene. These findings suggested that two H1N1 ts clones each had a ts lesion on the P1 gene. Unexpectedly, plaques were formed when cells were co-infected with clones ts-104 and ts-127 at restrictive temperature.

These results suggested that the two H1N1-ts reassortants exhibited intrasegmental complementation. The H1N1 ts progeny clones ts-104 and ts-127 were then genotyped; the ts-104 virus received only the NP and NS genes from the ts-1A2 parent and the ts-127 virus contained all H1N1 genes. Considered together, the genetic analysis and the observed genotypes suggested that the ts-104 and ts-127 viruses contained spontaneous ts mutations on the P1 gene. This was unambiguously shown to be the case by segregational analysis which identified the ts gene in the ts-127 and ts-104 viruses as the P1 gene. Thus, the P1 gene of the influenza A virus can sustain at least 2 complementing ts mutations suggesting that there may be at least two functional domains in this large polypeptide.

Further genetic analysis of the progeny from the A/Udorn/72 ts-1A2 (H3N2) virus X A/Hong Kong/123/77 (H1N1) virus cross revealed an interesting genetic interaction in which the H1N1 wild type nucleoprotein (NP) gene suppressed the ts phenotype specified by a ts P1 gene. As expected, each of the H1N1 ts reassortant viruses that received the ts-1A2 P1 gene failed to complement the HK/68-ts-315 virus (the ts parent from which the ts-1A2 ts P1 gene was derived). In addition, the HK/77-ts-1A2 reassortant ts-104 that contained a spontaneous ts P1 gene failed to complement the ts-315 virus. It was therefore expected that the ts-104 virus would not complement any of the H1N1-ts-1A2 ts-P1 reassortant viruses that contained the ts-1A2 P1 gene, but this was not the case. Two H1N1 ts-1A2 reassortant viruses that received the ts-1A2 ts-P1 gene complemented ts-104, whereas 2 others did not. The H1N1 ts-1A2 reassortant viruses that complemented the ts-104 virus received their NP gene from the H1N1 parent, whereas the others received an H3N2 NP gene. Genes at other loci did not affect complementation. The H1N1-ts-173 reassortant that contained the ts-1A2 ts-P1 gene and the H1N1 NP gene (complementation positive with the ts-104 virus) was mated with an H3N2 wild type virus and H3N2-ts reassortant viruses were sought that were complementation positive or negative with the ts-104 virus. Five H3 ts viruses have been analyzed to date and 3 were complementation positive with the ts-104 and each contained H1N1 NP gene, whereas the two complementation negative viruses contained an H3N2 NP gene. These findings suggest that a wild type NP protein (from an H1N1 virus in this instance) can suppress the ts phenotype specified by the P1 gene. The importance of these observations is two-fold. First, the data indicate that the product of the NP gene interacts functionally with the P1 protein; this further defines the interaction of proteins within the influenza RNA transcription complex. Second, the ts phenotype of a reassortant virus can be modulated not only by extragenic suppressor mutation but also by suppressor effects mediated by wild type gene products. This observation has important implications for influenza A virus vaccine development. The major implication is that reassortant vaccine viruses should contain all six internal genes from an attenuated donor virus in order to minimize the unpredictable phenotypic effects that could arise from suppression by genes from the wild type parent. This consideration would be especially important for reassortant vaccine viruses derived from a donor virus for which there is no simple laboratory marker of attenuation.

Evaluation of Host Restrictive (hr) Genes as Determinants of Attenuation of Influenza A Virus for Primates

In view of the genetic instability of the attenuating genes of the ts-1A2 donor virus and the genetic drift observed with two of the ca reassortants we initiated an alternate approach to attenuation of human influenza A viruses. This approach involves the use of an avian influenza A virus as the donor of attenuating genes. We sought avian influenza A viruses that were restricted in their replication in primate cells. We reasoned that the avian influenza genes responsible for restriction of growth in primate cells should attenuate reassortant viruses into which these genes were transferred. In this manner, it might be possible to prepare attenuated influenza virus reassortants bearing surface antigens of new epidemic or pandemic viruses. Thus, restriction would be effected by naturally occurring avian influenza virus genes rather than by mutant genes produced by chemical mutagenesis or selected by passage of virus at a low temperature or in an unnatural host.

Initially, 10 serologically distinct viruses recovered from asymptomatic birds were plaque purified by Dr. Virginia Henshaw, St. Jude Children's Hospital, Memphis, Tennessee and were then evaluated for evidence of restriction of growth in the respiratory tract of hamsters. The 10 viruses exhibited a spectrum of replication in the hamster's nasal turbinates ranging from viruses that replicated as efficiently as human influenza A viruses to viruses that were 8000-fold restricted. In squirrel monkeys the 10 avian influenza A viruses exhibited a spectrum of replication and virulence. The level of replication of virus and clinical response were closely correlated. Two viruses, A/Mallard/NY/78 (Hav7N2) and A/Pintail/79 (Hav1Neq2), resembled wild type human influenza A virus (H3N2) in level and duration of virus replication and in virulence. At the other end of the spectrum five avian viruses were restricted 100 to 10,000-fold in replication in the upper and lower respiratory tract and were clearly attenuated compared to human influenza virus. The avian gene or genes responsible for restriction of replication in primate cells can now be identified by the study of reassortants produced by mating a restricted avian virus with a virulent human influenza A virus.

Once the restricting genes and possibly incompatible human-avian gene constellations are identified, an appropriate donor virus can be constructed by genetic reassortment. Many of the influenza genes that have evolved over a long period of time in birds differ significantly from the corresponding genes of human influenza viruses when studied by nucleic acid hybridization. Because of these marked differences, it is likely that the avian genes will retain their attenuated characteristics after limited replication in man and thereby prove to be stable phenotypically.

Development of ELISA to Detect Influenza Antibody in Serum and Respiratory Secretions

We had previously described an ELISA that used whole virus as antigen to determine if a seroresponse occurs in volunteers receiving attenuated or wild type virus. However, in this assay, whole virus that is used as antigen that detects antibodies to both the subtype-specific surface antigens and type-specific internal antigens. Immunity to the hemagglutinin (HA) and neuraminidase surface glycoproteins correlates with resistance, whereas that to the internal type-specific antigens does not. Therefore, it was important to develop a sensitive ELISA that would detect antibody to the epidemiologically important surface antigens of the influenza A virus. To this end we developed an HA-specific ELISA that can be used to measure antibody in serum and nasal wash specimens. The ELISA employed 96-well plates and was carred out using the following ladder of reagents from the solid phase up. (1) purified hemagglutinin, (2) human serum or nasal wash specimen, (3) highly specific rabbit anti-human IgG, IgA, or IgM, (4) goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase, and (5) substrate.

In post-infection human sera HA antibody of the IgG isotype was predominant, whereas in nasal secretions HA antibody was predominantly IgA. The antibody response detected by the ELISA manifested hemagglutinin subgroup specificity. In addition, there was a good correlation between the ELISA antibody titer and the hemagglutination-inhibition or neutralizing antibody titer. The ELISA was more sensitive than the hemagglutination-inhibition assay, and the range of antibody in human serum measurable by ELISA was from less than 1:20 for children who had not experienced influenza infection to 1:400,000 for adults convalescent from a secondary infection.

These observations indicated that the ELISA was sensitive and specific for HA and was therefore used to examine the level of antibody in the serum and nasal wash specimens of children undergoing primary infection with an H3N2 or H1N1 ca reassortant virus. The ELISA was more sensitive than the neutralization assay in detecting antibody in nasal wash specimens. An anti-HA response in nasal wash was detected in the IgA subclass of 14 of 17 infected vaccinees. Surprisingly, 13 of the 17 vaccinees exhibited a local anti-HA response in the IgM subclass and 9 in the IgG subclass. The pattern of antibody response in the serum was that expected for individuals undergoing a primary infection. Each vaccinee developed an IgG antibody response and 16 of 17 developed an IgM response. A correlation was observed between the local production of ELISA IgA HA antibody in nasal wash and its appearance in serum. These results indicated that primary infection with an attenuated ca reassortant virus was efficient in stimulating systemic and local immunologic responses, a finding in contrast to parenteral immunization with inactivated antigen.

The immunological response in adults undergoing a secondary H3N2 virus infection was also characterized. The following ELISA HA responses were noted in 19 adult volunteers infected with wild type A/Peking/79 (H3N2) virus: serum responses; IgG-13/19; IgA-14/19; IgM-1/19; nasal wash responses; IgG-5/19; IgA-14/19; IgM-0/19. These results are in contrast to those seen during primary infection when a high frequency of IgM HA antibody responses was detected in serum and nasal wash specimens. This indicates that adults who undergo infection late in a subtype era usually fail to mount an IgM response. Thus the detection of specific antibody in this isotype should not be relied on for rapid diagnosis of severe influenza A virus infection in adults under these conditions.

We had previously suggested that with more sensitive tests to detect antibody to the influenza hemagglutinin it should be possible to determine the relative contribution of local and systemic immunity to resistance to influenza virus infection. This indeed appears to be the case. Preliminary analysis of response to infection with a ca reassortant virus demonstrated that the presence of local IgA antibody correlated with resistance to infection, a relationship not previously demonstrable by other serologic techniques.

Production of Antibody by Peripheral Blood Leucocytes In Vitro

Infection of man with either wild-type or attenuated influenza virus induces the production of antibody to influenza A virus. In order to examine this response at the cellular level, we studied 7 normal volunteers infected by intranasal inoculation with cold-adapted, temperature-sensitive attenuated A/Alaska/77 influenza (H3N2) virus. In vitro cultures of peripheral blood mononuclear cells (PBMC) were established with and without pokeweed mitogen (PWM) one day before and 6 and 27 days after inoculation and the culture supernatants assayed for class-specific antibody to influenza virus in a two-step ELISA assay. Before inoculation, cells from only 2 of the 7 volunteers made measurable IgG influenza antibody which was produced only when driven by PWM. Six days post-inoculation PBMC from 6 of the 7 volunteers spontaneously produced significant IgG antibody to influenza virus; the addition of PWM decreased the production in 5 of these 6 cultures. On day 27, none of the unstimulated cultures produced IgG antibody to influenza virus; however, 6 of the 7 cultures produced measurable IgG influenza antibody when driven by PWM. Cyclohexamide (15 ug/ml) inhibited both the spontaneous and PWM-driven antibody production by more than 95%. IgA influenza antibody could also be measured and the results on day 6 paralleled those of IgG antibody. However, on day 28 an IgA influenza antibody was not spontaneously released and could not be stimulated by PWM. In summary, circulating cells spontaneously secreting both IgG and IgA influenza antibody can be detected 6 days following localized infection with attenuated influenza virus. However, at 4 weeks, only IgG "memory" cells are found; these cells must be reactivated in order to produce influenza antibody. This technique can be used to assess the traffic of cells producing antibody to influenza virus after infection or immunization of man. These studies were done in collaboration with Dr. Robert Yarchoan and Dr. David Nelson, NCI.

HLA-restricted Cytotoxic Cells in Peripheral Blood of Man After Infection with Wild-type Influenza Virus

T lymphocytes that manifest H-2 restricted cytotoxicity confer resistance to challenge with wild type influenza A virus in mice. H2 restricted effector cells induced by infection with one influenza A virus are cytotoxic for target cells infected with influenza viruses belonging to different subtypes. Cells with similar *in vitro* properties have been detected in man after stimulation of peripheral blood leucocytes *in vitro* with influenza antigens. However, in humans undergoing infection with influenza virus, HLA restricted lymphocytes cytotoxic for targets infected with influenza A virus have not been previously identified. To detect this important cell population, we studied the cytotoxicity of peripheral blood leucocytes obtained from volunteers experimentally infected with influenza A virus against a panel of lymphocyte target cells infected with influenza A or B virus. The target cells were either HLA identical, partially related, or unrelated to the peripheral blood effector cells. Volunteers undergoing infection demonstrated HLA restricted cytotoxicity on days 6 and 10 post-infection that was specific for influenza A virus-infected cells but not for influenza B virus-infected cells. In addition, a transient increase in the numbber of NK cells was detected also on days 6 and 10. These results indicate that non-specific and virus-specific cytotoxic cell populations are stimulated after infection of man with influenza A virus. The identification of this important cell population will make possible the evaluation of their role in resistance to and recovery from influenza virus infections in man. This work was done in collaboration with Dr. G. Quinnan and J. Davies, BOB, FDA.

Characterization of Influenza A Virus Proteins Using Monoclonal Antibodies

Monoclonal antibodies have been produced previously to the hemagglutinin, neuraminidase, nucleoprotein, and matrix proteins of the influenza A virus. These antibodies have been used to characterize the number of antigenic sites on a protein and to examine the functions of the proteins, such as inhibition of polymerase activity. Studies initiated this year will focus on two major areas of research: (1) the practical usefulness of existing monoclonal antibodies to genotype recombinant viruses; to rapidly detect viral antigens in clinical specimens; to explore the antigenic specificities recognized by cytotoxic T-cells; and to probe the protein structure of the influenza A virion; and (2) the production of monoclonal antibodies to the polymerase and nonstructural proteins. Such monoclonal antibodies will be used to probe the antigenic and functional activities of these proteins. To these ends, the major efforts this year have been two-fold: in developing polymerase assays to detect transcapping, polymerization, and polyadenylation activities of the P proteins and in producing a sufficient quantity of polymerase proteins for immunization of mice. These objectives have been reached in part due to the the help of Connaught Laboratories which donated 50 liters of allantoic fluid containing a high titer of A/Bangkok/78 x 73 (H3N2) virus. The virus has been banded by Dr. John Gerin by continuous gradient centrifugation and we are presently isolating active RNP complexes from 120 ml of virus concentrated to contain 256,000 HA units/ml. These active RNP complexes should prove useful as antigen in the immunization of mice for the production of monoclonal antibody to the P proteins.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00026-14 LID

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October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
LABORATORY AND EPIDEMIOLOGIC STUDIES OF VIRAL HEPATITIS AGENTS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Richard J. Daemer, Ph.D.	LID, NIAID	Res. Microbiologist
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Bengt Hansson, M.D.	LID, NIAID	Visiting Fellow
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SECTION
HEPATITIS VIRUSES SECTION

INSTITUTE AND LOCATION
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

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SUMMARY OF WORK (200 words or less - underline keywords)

This project consists of continuing studies of the chemistry, structure, epidemiology, immunology and pathology of human and related hepatitis viruses. The foal of such studies is the control of human viral hepatitis by application of the most appropriate methods, including active and passive immunization, chemotherapy and interdiction of spread of the virus. Progress: Hepatitis A virus has been isolated directly from the stool of a patient into primary African green monkey kidney cell culture, a substrate suitable for vaccine development. The RNA genome of hepatitis A virus is being cloned in E. coli. Modifications in hepatitis B subunit vaccine have resulted in a highly immunogenic and safe preparation that produces a seroconversion in approximately 60% of vaccinees within two weeks of vaccination and in 95% following completion of the vaccination schedule. A "new" antigen, possibly reflecting the presence of a "transforming" gene, has been found in certain cells that contain integrated HBV genome. Non-A, non-B hepatitis agents have been transmitted to chimpanzees and, probably, marmosets, and ultrastructural changes that appear to be specific for non-A, non-B hepatitis have been identified. Evidence has been obtained for the existence of an epidemic form of non-A, non-B hepatitis in India. Attempts to transmit the virus to chimpanzees are currently in progress.

Background

The Hepatitis Viruses Section of the Laboratory of Infectious Diseases was founded in 1974 and was the outgrowth of studies on viral hepatitis that began in 1964. The goal of the section is control of viral hepatitis; approaches to achieving that goal include a mix of basic and applied research in virology, immunology and epidemiology. Heavy reliance is placed upon collaborative studies with scientists both within and without NIH; experience has shown that such utilization of expertise has been both highly productive and cost-effective. The section has made important contributions to our understanding of the hepatitis viruses. Following is a brief resume of recent and current research interest. Certain of these projects will be reported in greater detail by members of the Section.

Hepatitis A Virus

Hepatitis A virus (HAV) resembles most closely the picornaviruses: It is a 27 nm particle with cubic symmetry that consists of four polypeptides with molecular weights similar to (but not identical with) those of the picornaviruses and a single stranded linear RNA genome. However, it is more heat resistent than the picornaviruses, can be found at a wider range of buoyant densities and may have a somewhat smaller genome.

Isolation of HAV in Tissue Culture - HAV was recently isolated in tissue culture for the first time by Provost. Successful isolation followed 31 serial passages in marmoset monkeys before recovery in fetal rhesus kidney diploid cell culture. Still more recently Frosner recovered HAV directly from feces in Alexander hepatoma cells, a transformed human cell line that probably contains the complete hepatitis B virus genome. We have attempted to isolate HAV directly from clinical materials into a cell line suitable for vaccine development. These studies, under the direction of Dr. Richard Daemer, have utilized three different strains of HAV: The MS-1 strain studied by Krugman at Willowbrook, the SD-11 strain recovered by Dienstag from a Naval recruit in San Diego and the HM-175 strain supplied by Dr. Gust from Australia. When available, HAV-containing serum samples and fecal samples were both employed for isolation attempts. Immunofluorescence and, to a lesser extent, radioimmunoassay were used as indicators of virus multiplication. All three strains of HAV were serially passaged in marmosets up to 9 times, and marmoset-passaged virus was compared, at various passage levels, with HAV-containing clinical materials in isolation attempts. Although partially successful results were obtained with all three strains, consistently better results were obtained with the HM-175 strain, and subsequent studies were carried out only with this strain. Both marmoset passaged (pass 6) HAV and HAV in a fecal suspension were successfully isolated in primary African green monkey kidney (AGMK) cell culture. Multiplication of the virus at early passage levels was slow, requiring 8 or more weeks to achieve moderate to intense fluorescence in the cultures. RIA tests revealed that all of the detectable antigen was cell-associated. For this reason subsequent passage of the virus was carried out by passage of live cells. HAV is currently in 13th passage in primary AGMK, and the duration of each passage has shortened from approximately 8 weeks to 3-4 weeks. Virus isolated directly from feces is growing as well as marmoset-passaged virus. In contrast to the results obtained with the primary AGMK, similar attempts to isolate the HM-175 strain of HAV in WI-38 and MRC-5 cell lines were totally negative, even after 5 serial blind passages. We are currently attempting to further adapt HAV to grow in primary AGMK and to adapt it to growth in diploid cultures. These studies are being carried out by Dr. Mark Sobsey during a sabbatical year in our laboratory.

Cloning of HAV - Because HAV is difficult to grow in tissue culture and because a number of questions remain about is molecular virology and taxonomy, we have attempted to clone the HAV genome in *E. coli*. Such a cloned genome would be useful in studies of viral nucleic acid synthesis. Dr. John Ticehurst has purified HAV from infected marmoset livers and extracted viral RNA. Using a series of enzymatic steps he has prepared complementary DNA and from this has prepared double-stranded DNA consisting of strands complementary to positive and negative strands of the original RNA. This double-stranded DNA has been inserted into an appropriate plasmid and has been replicated in *E. coli.* Several clones of plasmid contain DNA that hybridizes with the original RNA preparation. Experiments are currently in progress to confirm that this clone, is, indeed, derived from HAV RNA.

Preparation of Monospecific (hybridoma) antibody to HAV - Virtually all antisera used for the diagnosis and characterization of HAV infection are convalescent sera obtained from marmosets, chimpanzees or man. In order to prepare more specific reagents Dr. Vincent McAuliffe has attempted to prepare hybridoma antibodies from mice hyperimmunized with HAV partially purified from marmoset liver. Several hybridoma clones that appear to synthesize antibody to HAV have been detected and are currently being purified and further characterized.

Radioimmunoassay for detecting IgM-class anti-HAV - Antibody to HAV is almost always present by the time clinical signs and symptoms appear, thereby making diagnosis of an acute infection difficult. However, tests that would permit detection of the IgM class of anti-HAV could be used for diagnosis of recent HAV infection with a single acute phase serum. Dr. Bengt Hansson developed a solid-phase radioimmunoassay during his stay in the Hepatitis Viruses Section under the Fogarty Visiting Program. The test is quite sensitive, detects antibody for approximately three months after onset of illness and requires only a small amount of serum.

Hepatitis B Virus

Hepatitis B virus (HBV) is a unique, 42nm virus of complex structure that contains a double-stranded circular DNA with a single-stranded gap spanning 20-50% of the genome. Recently three other viruses with similar characteristics have been discovered: The woodchuck hepatitis virus, (WHV), the Beachey ground squirrel virus and the duck hepatitis virus. Undoubtedly other members of this group will be discovered in other species. HBV is the cause of an enormous amount of morbidity and mortality, accounting for 30-50% of clinical hepatitis diagnosed in the U.S. However, it is in Asia and Africa where the virus causes the greatest damage, resulting in chronic infection of 5-10% of the population and leading to chronic hepatitis and hepatic cell carcinoma in a significant proportion of these individuals. For these reasons control of HBV is an important public health goal.

HBV Vaccine - Work has continued on the development and characterization of hepatitis B vaccine. Modifications made in the vaccine formulation have resulted in a highly immunogenic preparation, capable of stimulating antibody in a majority of recipients receiving as little as 5 ug of vaccine and producing a rapid and brisk antibody response: 60-80% of vaccinees develop antibody within one month of vaccination and 95% seroconvert by completion of the vaccination series. The NIAID vaccine produces antibody in approximately 2-3 times as many recipients by one month after vaccination as other vaccines currently undergoing testing in the U.S. For this reason we believe that the NIAID vaccine will be useful in those situations where rapid antibody response is essential, such as interruption of the maternal-fetal transmission of HBV that is common in Asia. Plans are underway to carry out an efficacy trial of the NIAID hepatitis B vaccine in such a setting.

"New" Antigen-antibody Systems Associated with HBV Infection - The possible etiological association between HBV infection and subsequent development of hepatic cell carcinoma (hepatoma) suggests the possibility that "neoantigens" may be present in HBV-infected cells or in hepatoma cells that contain the HBV genome. Traditionally, these have been sought by employing as an antibody reagent the serum from a tumor-bearing animal. Man and certain higher primates are the only species known to be susceptable to HBV infection, and only man has developed documented hepatoma (the similar woodchuck hepatitis virus and duck hepatitis viruses are both associated with hepatoma in their respective hosts). Thus, patients with hepatoma may serve as sources of antibody for a hypothetical HBV neoantigen. Dr. Rodney Johnson has tested sera from patients with hepatic cell carcinoma from Japan and South Africa for antibody that reacts with a human hepatoma cell line that contains the HBV genome but does not react with an HBV negative hepatoma line. He has found at least one antigen-antibody system that defines a nuclear antigen in the HBV-containing cells. Work is in progress to characterize the antigen and to determine if it is a product of the genome of the virus or of the host.

Integration of the HBV Genome into Host Chromosomal DNA - In a collaborative study with Dr. Gerin and Dr. Ding-Shinn Chen, we have sought evidence of integration of the HBV genome into chromosomal DNA from liver tissue of Asian patients with hepatoma. Such integration has been found at multiple and different sites within the high-molecular weight DNA extracted from hepatoma tissues. Surprisingly, integration was also found in non-tumor tissue obtained elsewhere in the livers of these patients with chronic HBV infection and hepatoma. Preliminary results obtained with liver tissue from chimpanzees with chronic HBV infection but without hepatoma suggested that integration may be an event that occurs in HBV infections long before transformation to hepatoma occurs. Additional studies of acute and chronic HBV infection in chimpanzees are under way to determine just how early in the infection integration occurs.

Woodchuck Hepatitis Virus - The similarities between HBV and WHV, coupled with the tendency for each virus to lead to chronic hepatitis and hepatic cell carcinoma in their respective hosts, makes WHV and its host, the woodchuck, a particularly interesting animal model system. In collaboration with Dr. Gerin and Dr. Popper, we have been studying the virology and pathology of WHV-associated hepatitis and hepatoma in woodchucks. Sensitive serologic tests have been developed for the WHV antigens and antibodies and attempts to transmit the virus to both woodchucks and primates are in progress. Dr. Popper has characterized the histopathology of acute and chronic hepatitis and hepatic cell carcinoma in animals that were naturally infected. These studies should not only better clarify the role of hepatitis viruses in the etiology of hepatic cell carcinoma, but may provide a means of predicting whether hepatitis vaccines will prevent hepatic cell carcinoma.

Non-A, Non-B Hepatitis Agents

As serologic tests for HAV and HBV infection are more widely applied it is becoming apparent that non-A, non-B hepatitis agents are widely distributed and associated with a significant proportion of clinical hepatitis cases, perhaps 25% of all clinical cases and as much as 90% of post-transfusion hepatitis. A number of such agents have been successfully transmitted to chimpanzees, but despite intensive efforts, an antigen-antibody system unequivocally specific for non-A, non-B infections has not been detected. An exception is delta antigen, an antigen associated with a unique non-A, non-B agent.

Delta Antigen- In 1977, Mario Rizzetto discovered a new antigen in patients with chronic type B hepatitis. This antigen, detected by fluorescence, was found in hepatocyte nuclei of patients with chronic type B hepatitis. Its distinctness from hepatitis B core antigen was demonstrated. Extensive studies with delta antigen were carried out by Dr. Rizzetto as a Fogarty Visiting Scientist in collaborative studies with Dr. Gerin's laboratory and the Hepatitis Viruses Section. Delta was found to be associated with a transmissible agent that required HBV synthesis for its own synthesis and that suppressed the synthesis of HBV markers. Thus, it had the characteristics of a defective interfering agent. Delta could be detected by immunofluorescence in the liver of chimpanzees experimentally infected with the agent but could not be detected in the serum until centrifugal pellets of the serum samples were treated with detergent. Thus, delta appeared to be enclosed within a particle. When samples of chimpanzee plasma containing delta were subjected to centrifugal separations, delta copurified with 35-37nm particles that resembled large HBsAg particles. Furthermore, these particles were coaggregated with the 22nm HBsAg particles by anti-HBs. Thus, delta was an internal component of particles incapsidated by HBsAg. When such particles were extracted for nucleic acid, a low molecular weight (approx. 500,000 daltons) piece of RNA was detected. This RNA also appeared to be enclosed, with delta, inside a coat of HBsAg. Delta could be readily transmitted to chimpanzees either acutely or chronically infected with HBV but could not be transmitted to HBV-immune chimps. However, electron micrographic changes similar to those seen during acute non-A, non-B hepatitis infection (see below) have been detected in such chimpanzees, suggesting that delta may share some characteristics in common with other non-A, non-B agents.

Dr. Rizzetto developed a radioimmunoassay for delta and anti-delta and performed seroepidemiologic studies. He found that delta had a world-wide distribution but that it was highly endemic in Italy, especially in southern

Italy, where it was associated with 50% of acute HBV infections. In Europe and the U.S. evidence of infection with delta was found most frequently in those extensively exposed to blood and blood products (hemophiliacs, illicit drug users, etc.). In summary, delta appears to be a unique transmissible agent with characteristics unlike those of any previously described self-replicating agent.

"Classical" Non-A, Non-B Agents - The epidemiology of the non-A, non-B agents resembles that of HBV quite closely: the agents are primarily transmitted by blood and blood products and lead to chronic infection in a high proportion of patients. Until recently (see below) the non-A, non-B agents were not associated with fecal-oral transmission or common-source outbreaks of hepatitis. Although satisfactory serologic tests have not been developed, Dr. Yohko Shimizu, while a Fogarty Fellow, did detect unique electronmicrographic changes in hepatocytes of chimpanzees experimentally infected with non-A, non-B agents. Two types of changes were observed, cytoplasmic changes associated with fusion of membranes of the endoplasmic reticulum into peculiar cylindrical structures and nuclear changes characterized by pycnosis, heterogeneous staining and, in a small proportion of cells, clusters of particles. In initial experiments, each of these electronmicrographic changes was seen independently, suggesting that they were caused by two different agents. Both changes have subsequently been found in chimpanzees inoculated with the same inoculum, and it is not yet clear whether the changes represent different stages of infection or whether the inocula contain more than one agent. Attempts to sort this out are currently in progress.

Dr. Stephen Feinstone has continued studies on the transmission of non-A, non-B agents to primates and has completed the infectivity titration of two strains, one that produces cytoplasmic changes and one that produces predominantly nuclear changes. The former has an infectivity titer of only approximately ten chimpanzee infective doses (CID) per ml; the latter has an infectivity titer of approx. 10^7 to 10^8 CID per ml. Such titered virus suspensions will now be used to begin the characterization of these agents.

In addition, Dr. Feinstone has successfully transmitted non-A, non-B agents of both types to marmosets. The nuclear changes were reproduced in marmosets, but the cytoplasmic changes have not been seen to date.

In collaborative studies with Dr. Harvey Alter and Dr. Jay Hoofnagle, liver biopsies from patients with chronic non-A, non-B hepatitis are being examined by electronmicroscopy by Dr. Hans Dienes. In several such biopsies he has found the peculiar nuclear particles that have been seen in chimpanzee and marmoset livers of animals infected with one type of non-A, non-B agent. Thus, certain electronmicrographic changes appear to be found consistently in various species infected with non-A, non-B agents.

Indian Hepatitis - In collaborative studies with Dr. Khorshed Pavri, we have found that two outbreaks of epidemic water-borne hepatitis and one cluster of cases of endemic hepatitis in India could not be serologically related to infection with HAV or HBV. Thus, these cases, which resembled type A hepatitis in many respects, appeared to be caused by non-A, non-B agents. It is not clear whether these agents represent one or more previously unrecognized serotypes of HAV or whether they are entirely new agents. Attempts to transmit them to chimpanzees and marmosets are currently in progress.

Other Studies

Anti-DNA Antibodies - Villarejos and others reported that patients with hepatitis of whatever etiology developed antibodies to single-stranded DNA during the acute phase of infection. Since detection of such antibody would be a useful tool for the study of pathogenesis of hepatitis as well as possibly a method of diagnosis for inapparent non-A, non-B hepatitis, we attempted to confirm this finding. Dr. Ian Gust, during his sabbatical stay in the Hepatitis Viruses Section, established several published assays for anti-DNA antibodies and compared results obtained from patients with other immune diseases to those in patients and experimentally infected chimpanzees with various types of viral hepatitis. He could not confirm the report that anti-single-stranded DNA antibodies developed routinely during acute viral hepatitis. Coded samples of serum from naturally infected humans and experimentally infected chimpanzees were distributed to the laboratories claiming success in the detection of such antibodies. The laboratories could not successfully break the code or agree between themselves even though they had published the original reports as collaborative studies.

Histopathology of Viral Hepatitis in Chimpanzees - In collaboration with Dr. Hans Popper, we have examined the similarities and dissimilarities of histologic changes occurring in the liver of chimpanzees experimentally infected with HAV, HBV or non-A, non-B agents. All samples were evaluated by Dr. Popper under code. Subtle but distinct differences in histopathologic lesions caused by the three viruses were detected.

Attempts to Prepare Cell Substrates Suitable for Isolation of Hepatitis Viruses - Of all the hepatitis viruses, only HAV has been successfully isolated and serially propagated in cell culture. Even this has been extremely difficult to achieve, and only a few isolation attempts have been successful. There is therefore a need for cell systems in which the hepatitis viruses can be readily isolated. Human and chimpanzee hepatocytes are, of course, susceptible to *in vivo* infection with all of the agents, but *in vitro* cultivation of primary hepatocytes is very difficult. Furthermore, viable liver tissue suitable for initiation of cultures is very difficult to obtain. Continuous hepatoma cell lines hold some promise: one such cell line that contains the HBV genome was successfully infected with HAV, but this is not a highly sensitive system. A potentially more useful approach is the development of hepatocyte hybridomas derived from fusion of differentiated hepatocytes with hepatoma cells that are immortal in culture. Dr. Lawrence Widman has demonstrated the feasibility of this approach with rat hepatocytes and rat hepatoma cells; the resultant hybridomas were shown to maintain many of the physiological characteristics of the hepatocyte parent but to be continuously cultivatable *in vitro*. In collaboration with Dr. Widman, Dr. Daemer is attempting to produce human hepatoma-chimpanzee hepatocyte hybridomas that will retain from the hepatocyte parent the receptor sites and metabolic pathways necessary for systhesis of the hepatitis viruses and from the hepatoma the ability to grow continuously in culture.

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BASIC STUDIES OF MYCOPLASMAS

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INSTITUTE AND LOCATION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These efforts covered both basic and applied aspects of mycoplasmas and related wall-free prokaryotes, including their neurotoxins, antigens, and other biological factors involved in virulence, their immunological interrelationships, and their possible role in human disease(s) of uncertain etiology. Current projects concern the characterization, pathogenicity, and serological properties of an expanding group of helical mycoplasma (spiroplasmas) being isolated from plants and a variety of insects (especially ticks). Experimental infections of suckling rats with a pathogenic spiroplasma (SMCA) has shown a variable occurrence of spongiform encephalopathy and no obvious inflammatory response in brain tissue, despite the appearance of central nervous system symptoms and as many as 10^8 organisms/gm of brain tissue. Helical spiroplasmas are not observed in brain sections examined by EM. This observation has obvious similarities to a number of "slow virus" infections of the human central nervous system. Culture medium developed in this section for spiroplasma isolation has been successfully employed in the recovery of a new mycoplasma from the human urogenital tract of patients with non-gonoccoccal urethritis. The organism possesses a unique specialized terminal structure that appears to play some role in attachment of the organism to tissue cells.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Studies on the biology and pathogenicity of helical mycoplasmas (spiroplasmas) have continued, with current efforts directed to: (1) their biological and serological characterization, (2) development of appropriate models to study pathogenicity, (3) distribution in hosts in contact with man, and (4) evaluation of various techniques (serology, isolation, etc.) to assess the possible role of spiroplasmas in acute and chronic human disease. These research efforts have resulted in important secondary consequences to classical mycoplasma research, since culture media developed in our spiroplasma studies has been successfully applied not only to the enhanced isolation of Mycoplasma pneumoniae (see previous report), but to the isolation of a new mycoplasma from the human urogenital tract.

(A) Spiroplasmas in central nervous system disease. The SMCA (suckling mouse cataract agent) group of spiroplasmas occurs in arthropods (ticks) and produces a syndrome in suckling rats that has characteristics of a number of "slow viruses," including Creutzfeld-Jakob (CJ) disease in man, scrapie in sheep and rodents, etc. SMCA spiroplasmas produce variable spongiform changes in brain (in absence of a histological response related to inflammation), persists for long periods (two-and-a-half years) in brain and other tissues, and has not been visualized in infected tissues by electron microscopy. Recently, helical-like filaments have been observed (by F.O. Bastian) in fixed brain of a patient with CJ disease. This observation has been confirmed now in at least six additional patients with CJ disease in the UK and the USA. Our present experimental studies are directed to a reproduction of a reliable animal model of the CNS disease, to identification and culture of spiroplasmas from infected tissues, and to visualize and identify tissue localization of the organism in brain. We have shown that intracerebral inoculation of as few as 3-30 spiroplasmas will result in a brain infection (in 15-20 days) where as many as 10^8 or 10^9 organisms/ gm brain tissue can be recovered in culture. Rather large numbers of spiroplasmas can be recovered for at least 90 days. The response appears to be a generalized infection of the brain, since about equal numbers of spiroplasmas can be recovered from the right or left cortex and slightly smaller numbers of organisms are recovered from cerebellum and brain stem. Work is currently underway to utilize fluorescent antibody and/or peroxidase staining techniques to identify areas of brain involved and to study various fixation techniques which would preserve the ultrastructure of the organisms. This model will also provide appropriate materials to compare the various biologic features (ether sensitivity, formalin effects, filtration data, etc.) of spiroplasma in brain tissue to the properties ascribed to CJ brain material and other brain encephalopathies. As an adjunct to this work, we are collecting brain tissue and serum from patients with CJ disease for cultivation attempts and for serological analysis with currently available spiroplasmas. (Bastian, University of Maryland).

(B) Genital mycoplasma research. Although non-gonococcal urethritis (NGU) in man appears to be frequently associated with some mycoplasmas (ureaplasmas) and chlamydia, considerable numbers of NGU cases cannot be related to the presence of either of these agents. Using the newly developed SP-4 culture medium for spiroplasmas which was formulated in this section for cultivation of the SMCA spirplasma, we have isolated two strains of a new mycoplasma from urethral specimens of two patients with NGU. The new mycoplasmas are unrelated to other mycoplasmas of man, are propagated only on SP-4 medium, and possess a unique terminal structure. The organisms adhere to glass, plastic, erythrocytes, and to cultured monkey kidney cells, producing cytopathogenic effects on the latter cells. One of the patients yielding the organism did not have culturable chlamydia, ureaplasmas, or other bacteria. We believe these early and preliminary observations suggest a strong relationship between the organism and tissue pathology, and possibly NGU. Further work is in progress to characterize the organism, determine its occurrence in normal individuals, and to assess its role in human disease. (D. Taylor-Robinson, London; R.M. Cole, NIAID).

(C) Other research and related programs: A number of other areas are covered in current research programs, including the following:

1) The occurrence of *M. pneumoniae* in throats of children and adults in the absence of circulating antibody, and the role of these strains in the epidemiology of the disease.

2.) Characterization of the neurotoxin of *M. neurolyticum* and the role of this toxin and other neurotoxins in the pathogenicity of mycoplasmas. This area also includes studies on the role of *M. pneumoniae* in human neurologic disease.

3) The occurrence of surface material on the specialized terminal structures of a number of mycoplasmas and its role in cell adherence and cytopathogenicity.

4) The development of improved techniques for the isolation, detection, and control of mycoplasma contamination of cell cultures, and specific interactions of mycoplasmas and cells which result in cellular changes or other effects that may relate to immunopathological responses.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00028-23 LID

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

STUDY OF RESPIRATORY VIRUSES AND MYCOPLASMAS IN VOLUNTEERS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI.	Brian R. Murphy, M.D.	LID, NIAID	Med. Off.
	Robert M. Chanock, M.D.	LID, NIAID	Chief

COOPERATING UNITS (if any)
Flow Laboratories, Rockville, Md.; Univ. of Maryland School of Medicine, Balto., Md., Univ. of Rochester, School of Medicine, Rochester, NY; Children's Hospital Natl. Med. Center, Washington, D.C., Center for Disease Control, Atlanta, Ga.

LAB/BRANCH
LABORATORY OF INFECTIOUS DISEASES

SECTION
RESPIRATORY VIRUSES SECTION

INSTITUTE AND LOCATION
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.5	2.0	5.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The A/Alaska/77 (H3N2) ca reassortant was satisfactorily attenuated, genetically stable, and non-transmissible in seronegative adults. Its human infectious dose 50 was less than $10^{5.7}$ TCID$_{50}$, however, it was not possible to infect more than 75% of volunteers when 10-100 fold more virus was given. Refractoriness of a subset of volunteers to a large dose of virus appeared to be mediated by immune factors. Individuals who were infected with 10-100 HID$_{50}$ of virus lacked detectable nasal wash IgA antibody (1:2) to hemagglutinin, whereas refractory volunteers has a pre-existing antibody titer of 1:8 or greater. Another ca reassortant, the A/Cal/78 (H1N2) ca reassortant, was also attenuated at a dose of $10^{7.0}$ TCID$_{50}$. Thus, transfer of all six internal genes from the ca donor strain A/AA/6/60 appears to reproducibly attenuate influenza A virus for adults.

Project Description

Evaluation of Cold-adapted Reassortants in Adults - The influenza A/Ann Arbor/6/60 cold-adapted (*ca*) virus is being evaluated as a donor of attenuating genes to new antigenic variants of influenza virus. In previously studies, *ca* reassortant viruses that were derived from the A/Ann Arbor/6/60 *ca* donor virus were found to be attenuated for animals and man. Studies were carried out this year with an A/Alaska/77 (H3N2) *ca* recombinant. This recombinant possessed all six transferable A/AA/6/60 genes (i.e., genes other than those coding for the surface glycoprotein hemagglutinin and neuraminidase antigens). The A/Alaska/77 *ca* recombinant was previously found to be satisfactorily attenuated and antigenic in seronegative adult volunteers at a dose of $10^{7.7}$ $TCID_{50}$. However, only 18 of 24 volunteers were infected at this dose. Since many of these 24 vaccinees had presumably been infected with earlier H3N2 variants, it was possible that immunity indiced by prior infection was responsible for the observed resistance to the *ca* recombinant. To investigate this, two additional groups of volunteers were administered $10^{6.7}$ (12 volunteers) or $10^{5.7}$ (10 volunteers) $TCID_{50}$ of virus intranasally. Illness was not observed in the 22 volunteers and the virus shed retained the *ts* phenotype. Approximately 70% of the volunteers were infected at each dose. To determine if previously unrecognized immunity was responsible for this flat dose-response curve, the pre-inoculation nasal wash IgA hemagglutinin antibody titer to the H3-77 hemagglutinin was measured by ELISA and correlated with response to virus challenge. Twelve of 13 vaccinees with nasal wash an ELISA IgA anti-H3 hemagglutinin titer of $\leq$1:2 were infected, whereas only 3 of 9 vaccinees with a titer $\geq$1:8 were infected (p .007, Fisher exact test). These results suggest that specific immunity was responsible for the refractoriness of the subset of volunteers who failed to become infected with 10-100 HID_{50} of virus. Presumably this type of immunity would also modulate clinical response to wild type virus and thus the failure of the vaccine candidate virus to ifect all volunteers should not be viewed as a defect in this approach to immunoprophylaxis. It is encouraging that the estimated human infectious dose 50 (HID_{50}) of this H3N2 virus may be as low as $10^{5.0}$ $TCID_{50}$ (or less). Thus a vaccine dose of $10^{7.0}$ $TCID_{50}$, representing at least 100 HID_{50}, did not cause significant illness. Lower doses of $10^{4.7}$ and $10^{3.7}$ will be evaluated to define more precisely the HID_{50} of this vaccine virus.

We have also begun an evaluation of the A/California/10/78 *ca* reassortant virus and its wild type parent. The A/Cal/10/78 *ca* reassortant virus also possesses the six transferable A/AA/6/60 genes. These preliminary studies have been done in collaboration with Drs. Douglas and Betts at the University of Rochester School of Medicine in New York and Dr. Clements at the University of Maryland. The wild type virus was virulent in seronegative adults. The *ca* reassortant was satisfactorily attenuated in 23 vaccines when given at a dose of $10^{7.0}$ $TCID_{50}$. Immunologic response of these volunteers and genetic characterization of their isolates are currently under study.

A more current H3N2 virus, the A/Peking/2/79 (H3N2) wild type virus and a *ca* reassortant virus derived from it were evaluated in susceptible volunteers.

The wild type virus was not as virulent as the previous H3N2 wild type viruses we evaluated. Only 2 of 19 volunteers infected with $10^{4.0}$ to $10^{6.0}$ $TCID_{50}$ of virus developed illness (afebrile systemic illness). Previously, 50% of volunteers given $10^{4.0}$ to $10^{5.0}$ $TCID_{50}$ of one of 5 different H3N2 wild type viruses developed illness. This indicates that the wild type A/Peking/2/79 wild type virus was partially attenuated for man and thus could not be used as a challenge virus. Since the genetic determinants of this decreased virulence are not understood, but could reside in the genes coding for the glycoprotein surface antigens, extensive studies with *ca* reassortant viruses that possess the A/Peking/79 H3N2 antigens are not anticipated.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00175-04 LID

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
LABORATORY STUDIES OF PARAMYXOVIRUSES AND RESPIRATORY SYNCYTIAL VIRUS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI.	Robert M. Chanock, M.D.	LID, NIAID	Chief
	Gregory A. Prince, Ph.D.	LID, NIAID	Microbiologist
	Susan B. Spring, Ph.D.	LID, NIAID	Res. Biologist
Other:	Mark D. Tolpin, M.D.	LID, NIAID	Res. Assoc.

COOPERATING UNITS (if any)
Flow Laboratories, Rockville, Md. (Contract # N01 AI 32521); Meloy Labs., Rockville, Md.; Children's Hosp. National Medical Center, Washington, D.C.; NINCDS

LAB/BRANCH
LABORATORY OF INFECTIOUS DISEASES

SECTION
RESPIRATORY VIRUSES SECTION

INSTITUTE AND LOCATION
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	2.5	3.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

RS virus infection have concentrated on three approaches, namely, temperature-sensitive mutants, cold-adapted mutants, and bovine respiratory syncytial (BRS) virus. The most attenuated of the ts mutants was ts-2 (complementation group B). This mutant did not induce any symptoms but unfortunately it had low infectivity for seronegative chimpanzees and young children. Despite many passages at low temperature (24-26°C) cold-adapted mutants, produced in our laboratory and in the Sandoz Laboratories (Austria), retained virulence for chimpanzees equivalent to that of wild type virus. Bovine RS (BRS) virus, a naturally-occurring bovine pathogen, was not pathogenic for experimental rodents or primates. Studies in owl monkeys and chimpanzees, however, showed the virus to have very low infectivity, and limited immunogenicity.

Background

RS virus is the major etiologic agent of bronchiolitis and pneumonia of early life and the need for effective immunoprophylaxis has been clearly established in studies performed throughout the world. The challenge of vaccination against RS virus disease is formidable since the major impact of the virus is expressed during the first half year of life. This means that RS virus vaccine must be administered shortly after birth if it is to be effective in preventing the major share of serious RS virus disease. Unfortunately, this is a difficult period in which to attempt immunization since the infant's immunologic processes are not fully responsive to antigenic stimulation. In addition, antibody in serum that is passively acquired from the mother may act to suppress the infant's systemic immunoglobulin response.

Previously it was observed that a highly antigenic, inactivated RS virus vaccine did not provide protection against infection or disease caused by this agent. In fact, inactivated vaccine given parenterally induced an altered state of reactivity so that vaccinees developed more serious lower respiratory disease than usual when they underwent infection. In contrast, naturally acquired RS virus infection confers a definite degree of protection against subsequent RS virus illness. Observations made during a study of RS virus infection in adult volunteers suggested that local defense mechanisms in the respiratory tract play a major role in resistance to illness caused by the agent. For this reason, an attenuated strain of RS virus was sought that would induce resistance without producing significant illness.

(1) Temperature-sensitive mutants

Initially a series of ts mutants of RS virus were produced by chemical mutagenesis with the expectation that their temperature sensitivity would restrict growth in the target organ, the lungs. Studies in hamsters, an animal that has the same body core temperature as man (37°C), indicated that the RS ts mutants were restricted in replication in the lungs. Subsequently, one of the mutants, ts-1, was shown to be attenuated and highly antigenic in seronegative children. Infection with the ts-1 mutant did not produce any disease manifestations in the lower respiratory tract, however, seronegative vaccinees developed afebrile rhinorrhea indicating that the mutant retained a small amount of residual virulence. In addition, a few vaccinees shed small amounts of virus that produced plaques at restrictive temperature, i.e., ts^+ virus, indicating that the mutant was not completely stable genetically. Although this genetic instability was a cause for concern, two observations served as a source of encouragement. First, the ts-1 mutant was attenuated, but still highly antigenic. Second, there was no evidence that infection of seronegative individuals with the mutant induced a state of altered reactivity to subsequent infection with RS virus. Based upon these experiences efforts were initiated to identify a genetically stable mutant that would infect seronegative infants without producing rhinitis.

The group B mutant, ts-2, was of particular interest since it did not fuse cells to form syncytia during plaque formation at permissive temperature.

Recent studies indicated that this plaque morphology mutant had a defect in adsorption and/or penetration at restrictive temperature. It is likely that the ts defect of ts-2 affects the viral surface glycoprotein responsible for cell fusion and presumably also for viral penetration. The ts-2 mutant proved to be completely attenuated for susceptible chimpanzees. In addition, the mutant had low infectivity for chimpanzees.

In recent clinical trials by Drs. Wright, Karzon (Vanderbilt University), Parrott and Kim (Children's Hospital National Medical Center, D.C.) who administered this virus the ts-2 mutant was observed to be over-attenuated when it was administered to seronegative young children. Fewer than 1/3rd of the seronegative children given the maximum amount of virus possible ($10^{6.0}$ pfu) became infected. Although this mutant did not cause any symptoms in those children who were infected its low infectivity disqualifies it for use in immunoprophylaxis.

(2) Cold-adapted virus.

Attenuation of other viruses by repeated passage at low temperature suggested that this might be an effective approach with RS virus. We passaged the ts-1 mutant over forty times at 24°C, but found that chimpanzees inoculated with the high passage virus developed disease as severe as chimpanzees inoculated with the parental strain of virus. In addition to our efforts in this area, the Sandoz Laboratories, using a different parent strain, passaged virus repeatedly at low temperature. In collaboration with them, we inoculated this virus into chimpanzees, and found that it, too, was as virulent as wild type RS virus.

(3) Bovine RS virus.

Since 1971 a number of RS virus strains resembling human RS virus have been isolated from cattle with respiratory disease. The bovine RS (BRS) virus was subsequently shown to be ubiquitous among cattle herds throughout the world. Although it is antigenically related to human RS virus, it is distinct when tested by plaque reduction assay. Antiserum to the BRS virus neutralizes human RS virus at a titer approximately 2-4 fold lower than homologous virus, whereas, antiserum to human RS virus exhibits very little neutralizing capacity toward BRS virus. The efficacy of cowpox virus in the immunoprophylaxis of smallpox led us to consider the possibility of using BRS virus to prevent RS virus disease in man. Initial studies in the cotton rat showed that the virus infected this rodent, but did not produce histopathologic lesions. Furthermore, prior infection with BRS virus induced substantial resistance against challenge with human RS virus, particularly in the lungs. Based on these promising studies, we inoculated owl monkeys and chimpanzees with BRS virus. We found the virus to be considerably less infectious for these primates than for cotton rats. Significantly, BRS virus did not protect owl monkeys or chimpanzees from disease when they were challenged with human RS virus. In an effort to increase the level of replication of BRS virus in primates we passaged the virus rapidly in African green monkey kidney cells and observed a gradual adaptation to these cells. This BRS virus now grows to high titer and produces more prominent cytopathologic effects. In a study currently in progress in owl monkeys, the infectivity and immunogenicity of this monkey cell culture adapted mutant of BRS virus is being compared with that of the parental strain.

Immunity Studied Through the Aid of Parabiosis

Little is known of the immune factors responsible for active immunity to RS virus infection. There is evidence that secretory antibody is effective in preventing nasal infection, but no certain role for serum antibody, or cell mediated immunity has yet been described. We have used parabiosis as a tool to study the factors of active immunity. When an immune cotton rat (rendered immune by prior intranasal inoculation with live virus) is surgically linked to a non-immune animal, cross-circulation develops within one week. When the non-immune animal is challenged intranasally, it shows complete immunity to pulmonary infection, but no immunity to nasal infection. Thus, there is a systemic component of immunity to RS virus infection, that is transmitted through the blood and active only in the lungs . Future studies will seek to determine the factor or factors present in the blood that confer this immunity.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00179-03 LID

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

MOLECULAR BIOLOGY OF RESPIRATORY AND GASTROINTESTINAL VIRAL PATHOGENS STUDIED BY DNA RECOMBINANT TECHNOLOGY

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH LABORATORY OF INFECTIOUS DISEASES

SECTION MOLECULAR VIRAL BIOLOGY SECTION

INSTITUTE AND LOCATION NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We devised a procedure to obtain cloned DNA of influenza viral RNA sequences. Influenza viral gene segments that code for the hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein (M), non-structural proteins (NS) and one of the three polymerase proteins (Pi) were cloned in pBR322 of E. coli. Results from sequence analysis of several DNA clones provide evidence that cellular RNA sequences are utilized to prime influenza viral mRNA transcription in infected cells. Mapping of mRNA species as probed by the cloned NS DNA showed that two separate NS mRNA's are responsible for the NS1 and the NS2 polypeptides: the NS1 mRNA is a continuous transcript and the NS2 mRNA contains an interruption. Similarly, mapping of M mRNA transcripts revealed the presence of a colinear mRNA and two different interrupted mRNA's. These findings indicate that the M RNA potentially codes for 3 different polypeptides. Also, an HA-SV40 hybrid virus was constructed for expression of functional activity of the hemagglutinin in primate cells. The putative hemagglutinin produced in this system was glycoslyated and exhibited hemagglutination activity. Furthermore, the hemagglutinin product of the hybrid virus was detected on the surface of infected cells.

PHS-6040
(Rev. 10-76)

Objectives

(1) To employ recombinant DNA technology to study the genes of influenza A virus and their functions. (2) To elucidate the pattern of antigenic variation among influenza virus strains by analysis of cloned DNA sequences that code for the viral surface antigens. (3) To study the mechanism of viral gene expression at the transcriptional level by nucleotide sequence determination of cDNA copies derived from viral mRNA. (4) To examine cloned DNA for viral protein synthesis in eukaryotic cells as well as in E. coli (harboring influenza virus genes). (5) To determine the antigenic determinants in relation to antigenic variation and to delineate the functional domains of the viral surface proteins. (6) To develop procedures for conversion of cloned viral DNA back to the virion RNA with the intent of constructing and isolating defined mutants for use in experimental studies and possibly, for immunoprophylaxis.

Methods

(1) Preparation of influenza viral RNA segments - extraction of influenza virion RNA, fractionation of cytoplasmic viral mRNA from virus-infected cells.

(2) Synthesis of influenza viral cDNA strands - reverse-transcription of the virion RNA segments using a specific DNA primer which is complementary to the conserved 3'-end of virion RNA segments and reverse-transcription of the cytoplasmic viral mRNA segments using oligo (dT) as a primer.

(3) Cloning influenza viral DNA segments in E. coli plasmid pBR322.

(4) Characterization of cloned viral DNA molecules by restriction enzyme cleavage.

(5) Analysis of nucleotide sequence using the method of Maxam and Gilbert and the method of Maat and Smith.

(6) Immunoprecipitation and/or radioimmunoassay for viral peptides synthesized in E. coli containing influenza virus genes or in eucaryotic cells transfected with cloned viral DNA.

(7) Introduction of cloned viral DNA into mammalian cells by transfection using deletion SV40 DNA as a vector and by co-transformation using the thymidine kinase gene from herpes simplex virus.

(8) Conversion of cloned viral DNA back to influenza virion RNA - "marker rescue" of defined ts mutants of influenza virus by cloned DNA and sero-selection of a different influenza subtype using cloned DNA coding for the surface antigens.

Background

The genome of influenza virus consists of eight separate RNA segments that vary from 900 to 2400 nucleotides. The virion RNA segments are negative strands, that is, their complementary RNA transcripts become associated with the polyribosomes and are translated to produce viral specific proteins. With the exception of viral RNA segment 8 which encodes two different non-structural proteins and viral RNA segment 7 which encodes the matrix protein and possibly two additional short polypeptides, each of other RNA segments seems to code for a specific viral component and to be responsible for a specific viral function. The functional role which these viral proteins play in viral replication and maturation is only partially understood. Complementation-reassortment analysis of temperature-sensitive (ts) mutants of influenza virus demonstrates that at least eight complementation groups of ts mutants have been isolated and characterized.

One feature distinguishing influenza virus from other non-oncogenic RNA virus is that the functioning of the host nuclear RNA polymerase II is required for virus replication, especially for viral RNA transcription. Studies using an in vitro assay system show that capped eukaryotic mRNA's donate their 5'-terminal methylated cap and a short stretch of internal nucleotide to the 5' end of the viral RNA transcripts. Although recent results suggest that a similar mechanism appears to operate in vivo, more direct evidence for the host RNA involvement in the initiation of influenza viral mRNA transcription is still lacking. In addition, the sequence specificity and the length of the transferred RNA segment for the initiation of mRNA transcription are not known. Many questions relating to the regulation of transcription such as the biosynthesis of the two different mRNA's derived from a single viral gene segment (gene 8, the smallest RNA segment) remain to be answered.

Another important feature of influenza virus is antigenic variation, or the emergence of viral subtypes (antigenic shift). Other phenomena involving antigenic variation of a lesser extent within a subtype (antigenic drift) are also observed. Such antigenic instability results in epidemiologically significant differences in surface antigens (hemagglutinin and neuraminidase) seen in the influenza A virus subtypes. When this type of change is abrupt and extensive (antigenic shift) a pandemic ensues. As a consequence, the continuing change of influenza strains has rendered ineffective efforts to vaccinate man against major new antigenic variants.

In view of the medical importance of influenza viruses there is a need to broaden our knowledge of the genetics of these viruses in order to design ways to prevent pandemics. One approach is to use recombinant DNA technology that has been well-developed and has proven valuable in elucidating gene organization and expression in other appropriate host systems. Our general plan for this approach includes two phases. The first phase involves cloning each of the eight influenza gene segments using the E. coli K12-plasmid system (approved MUA #91). The second phase of development involves the use of purified clones of influenza DNA sequences to examine viral gene expression in animal cell culture and to produce vRNA in eukaryotic cells. To carry out this second phase we propose to utilize defective SV40 (lacking the late region of the geonme that codes for capsid proteins) for construction of influenza-SV40 hybrid DNA molecules (approved MUA #110). Introduction of the appropriate influenza-SV40 hybrid DNA molecules into eukaryotic cells may lead to transcription or translation of influenza viral DNA depending upon its orientation of insertion. Our goal is to devise procedures which would permit conversion of influenza DNA back to an influenza negative strand RNA and eventually transfer such RNA into an influenza virus. In this manner, stable site-specific mutations, such as deletions, induced in the cloned DNA might be transferred back to the influenza virus. Thus, it may be possible to develop stable mutants for experimental study and for use in immunoprophylaxis.

Major findings

1. Cloning DNA sequences from influenza viral RNA segments

We developed procedures to obtain DNA clones containing complete sequences of influenza A virus. Initially, cDNA strands were separately synthesized by reverse-transcription of genomic RNA segments using a specific dodeca-deoxyribonucleotide complementary to the 3'-terminus as a primer and by reverse-transcription of cytoplasmic viral mRNA using oligo(dT) primer. DNA duplexes were formed from the corresonding cDNA copies and inserted into the PstI site of pBR322 for transformation of E. coli. Also, DNA duplexes corresponding to all eight genomic RNA segments were produced by reverse transcribing (+) cDNA (prepared as described above) using another synthetic oligonucleotide complementary to the 3' end of (+) cDNA. These influenza viral DNA copies, expectedly full-length or almost full-length, were joined to the pBR322 vector using a defined oligonucleotide linker that contained a specific restriction enzyme site. The constructed recombinant DNA was subsequently cloned in E. coli. So far, DNA clones derived from six of the eight gene segments of influenza virus (A/Udorn/72 [H3N2]) were obtained. These six gene segments code for the hemagglutinin, neuraminidase, nucleoprotein, matrix protein, non-structural protein and one of the three polymerase proteins. The influenza virus-specific DNA sequences isolated from recombinant plasmid molecules were characterized by mapping restriction enzyme cleavage sites. In addition, the strand orientation of cloned DNA was determined with reference to the 3'-terminus of genomic RNA.

2. Non-viral sequences in cloned full-length influenza viral DNA

Our cloning approach also provided a strategy for analysis of additional 5'-terminal nucleotides in influenza viral mRNA species. Sequence analysis of terminal nucleotides of several independently isolated DNA clones showed that additional nucleotides were present between the dG/dC linker used for insertion of influenza DNA into pBR322 and the specific dodecanucleotide primer. The sequences of these inserts varied in length from 6 to 14 nucleotides and were heterogeneous in base composition among clones from the same gene and as well as from different genes. The heterogeneity and the ubiquity of these inserts at the 5'-terminus of mRNA suggest that these nucleotides were derived from cellular RNA species and functioned in the initiation of viral mRNA transcription. These findings provided direct evidence that cellular RNA molecules are utilized in priming viral mRNA transcription in infected cells. Two of eight clones exhibited an alteration of the first nucleotide of the primer. This frequent type of alteration suggests that mismatch correction had occurred and that transcription of influenza mRNA may commence with the second nucleotide at the 3'-terminus of vRNA. Several clones contained the entire conserved nucleotides at both termini suggesting that these cloned DNA's are complete copies of genomic RNA. These cloned DNAs, therefore, could potentially produce full-length copies of viral RNA that contain all of the control signals for viral gene expression in eukaryotic cells (see Major Findings, item 4, below).

3. Overlapping genes and interrupted mRNA's of influenza virus

Influenza viral RNA segment 8 codes for two unique polypeptides, NS1 and NS2, that are translated from separate mRNA's. Cloned full-length NS DNA was employed to map these mRNA's on the vRNA segment. These results showed that the body RNA for NS2 mRNA was 340 nucleotides and that for NS1 mRNA was 860 nucleotides. The two mRNA's share the same poly(A) addition site. Sequencing studies showed that NS1 mRNA is a co-linear transcript and the NS2 mRNA contains an interrupted sequence. The nucleotide sequences at the junctions of the interrupted segment are similar to those of the consensus sequences at the splice sites of intervening regions in eukaryotic mRNA's. The sequence analysis further showed that NS1 and NS2 share 9 common amino acids translated from the same initiation codon and diverge in amino acid sequences subsequently as a result of the interruption. More recently, cloned complete DNA from RNA segment 7 coding for the matrix protein was also employed to map M mRNA species. The results showed that in addition to the colinear transcript a small body RNA segment of 280 nucleotides was present. Sequence analysis showed that there are two separate small M mRNA's present, each containing an interrupted sequence at a different location. Sequence analysis indicates that translation of one of these small M mRNA's yields a sequence of 97 amino acids in a +1 reading frame and translation of the other mRNA yields a polypeptide of nine amino acids. These findings, from studies performed in collaboration with Dr. Robert Lamb of Rockefeller University, indicate that RNA segment 7 potentially codes for 3 different polypeptides.

4. Functional expression of cloned DNA coding for the hemagglutinin

We employed SV40 DNA as a vector for expression of functional activity of a cloned influenza viral DNA segment in eukaryotic cells. As a first step we attempted to produce the hemagglutinin surface antigen (HA) using the cloned complete HA DNA. These experiments included: (a) construction of SV40-HA recombinant DNA, (b) propagation of SV40-HA hybrids in the presence of a ts SV40 helper in AGMK cells, and (c) analysis of infected cells for production of hemagglutinin. In one experiment we observed that a clone of SV40-HA hybrid virus produced a polypeptide similar in molecular size to the hemagglutinin of influenza virus as indicated by immunoprecipitation and gel electrophoresis. Similar to the hemagglutinin of influenza virus the polypeptide product of HA-SV40 contained glycosyl residues as revealed by incorporation of radioactive labelled sugars. The putative HA product exhibited functional activity as shown by agglutination of guinea pig red blood cells. In addition, an indirect immunofluorescence assay and absorption of red blood cells on the infected cell surface showed that the hemagglutinin polypeptide of the hybrid virus was expressed on the surface of infected cells.

Proposed Course of Study

1. Cloning DNA sequences from the polymerase genes of influenza virus

To elucidate individual gene functions and their interaction we plan to obtain a complete set of DNA sequences from all eight gene segments of influenza virus. Similar procedures will be followed to obtain and characterize DNA clones coding for the 3 polymerase proteins. When this is accomplished, we will use the cloned DNA to examine sequences of these polymerase genes and their coding capacities. These DNA clones should also be useful for probing viral sequences in order to elucidate the mechanism of transcription and replication.

2. Polypeptide synthesis from cloned DNA of influenza virus

Parallel to the functional expression of cloned HA DNA in mammalian cells using SV40 vectors, we intend to achieve polypeptide synthesis and functional activity of all other cloned DNA of influenza virus. These experiments will allow us to determine the domains of each individual gene at the peptide level and elucidate peptide-peptide interactions among viral gene products. Influenza gene functions provided by cloned DNA's should also be valuable in biological experiments such as complementation of defective influenza virus mutants. In this manner, it may be possible to generate mutants defective in a specific function.

3. "Marker rescue"--conversion of cloned DNA back to virion RNA

In addition to functional expression of viral polypeptides full-length cloned DNA should be also valuable for conversion of cloned DNA back into virion RNA. SV40-influenza DNA hybrid viruses that synthesize the (+) or (-) full-length strand of influenza viral RNA transcripts will be constructed as described earlier. "Marker rescue" of influenza virus of different serotypes under antibody selection will be performed using SV40-HA recombinant virus that produces either the (+) strand or (-) strand of influenza viral RNA in a coinfected cell. Similarly, "marker rescue" of influenza virus ts mutants will also be attempted using a recombinant virus containing SV40 sequences and the cloned DNA of an influenza gene that corresponds to the ts gene of the ts mutant. If this procedure can be accomplished one could convert cloned influenza DNA back into genomic influenza RNA. Success in this approach would also enable one to construct influenza virus mutants containing strategic mutation sites introduced at the DNA level. Such mutants should be valuable for use in immunoprophylaxis and for biological studies.

Publications

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Lamb, R.A., Lai, C.-J. and Choppin, P.W. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: Colinean and interrupted mRNAs coded for overlapping proteins. Proc. Nat. Acad. Sci. (USA), in press.

Sveda, M.M., and Lai-C.J. Functional expression in primate cells of cloned DNA coding for the hemagglutinin surface glycoprotein of influenza virus. Proc. Nat. Acad. Sci. USA, in press.

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LABORATORY OF MICROBIAL IMMUNITY

1981 Annual Report
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PHS-NIH
SUMMARY STATEMENT

ANNUAL REPORT OF THE LABORATORY OF MICROBIAL IMMUNITY
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
October 1, 1980 to September 30, 1981

Richard Asofsky, M.D.
Chief, Laboratory of Microbial Immunity

Research in this Laboratory is concerned mainly with the differentiation of lymphoid cells and with basic mechanisms controlling cellular and humoral immune responses to microbial and tissue antigens. Blood parasites, bacterial polysaccharides, and lipopolysaccharides, as well as "integrated" leukemia viruses and histocompatibility antigens are used. Genetic studies on genes controlling lymphocyte differentiation, genes required for the expression of antigens of leukemia viruses, and genes controlling certain antibody responses are in progress. Neoplastic B-cell lymphomas are used to examine various stages of B cell differentiation. Somatic cell hybrids (hybridomas) are made to several groups of antigens. Two of the B cell lymphomas have been drug-marked, and are used to obtain B cell hybrids. Autoimmune diseases, such as the SLE-like syndrome of NZB mice and experimental encephalomyelitis in guinea pigs are under active study.

TREATMENT WITH ANTI-IDIOTYPIC ANTIBODY LEADS TO EXPRESSION OF A SILENT CLONE

BALB/c mice immunized with bacterial levan (BL) produce an immune response that fails to generate antibody expression to the idiotype (Id) of the B(2→6) fructosoan-binding myeloma protein ABPC 48 (A48). Pretreatment of newborn BALB/c mice (at one day of age) with 0.01-10μg of affinity-purified BALB/c anti-A48Id antibody followed by immunization with BL 1-2 months later produces an anti-BL response that expresses the A48Id. This shows the A48Id$^+$ anti-BL clones belong to a normally silent fraction of anti-BL repertoire. The activation of A48Id$^+$ anti-BL clones by anti-A48Id antibody was found to be specific and to require subsequent immunization with BL. (Drs. Baker, Hiernaux, Prescott, Bona, DeLisi and Rudbach).

LOW-DOSE PARALYSIS MEDIATED BY THYMUS-DERIVED SUPPRESSOR CELLS

Prior treatment (priming) with a sub-immunogenic dose of Type III pneumococcal polysaccharide (SSS-III) results in the development of an antigen-specific long-lasting form of unresponsiveness termed low-dose paralysis. The results of recent studies show that such unresponsiveness can be transferred by viable spleen cells obtained from mice within 5-24 hours after priming; the suppressive activity of primed cells is abolished by treatment with monoclonal antibody specific for the Thyl.2 alloantigen, an antigen characteristic for thymus-derived cells. These findings provide direct evidence for the involvement of suppressor T cells in the expression of low-dose paralysis. (Dr. Baker, Mr. Stashak, Ms. Amsbaugh, Mr. Caldes and Prescott).

DYSFUNCTION OF LYMPHOCYTES FROM NZB MICE: GENETIC STUDIES

NZB mice exhibit defects in T and B cell function which can be detected during the first month of life. NZB B lymphocytes undergo spontaneous polyclonal activation characterized by a decrease in plasma cell frequency and an elevated rate of IgM secretion/plasma cell. As all of these defects can be detected in the absence of T lymphocytes, they are felt to represent intrinsic abnormalitites of NZB B cells. NZB mice also exhibit a number of T lymphocyte dysfunctions. Included among these is an unexpected primary cyctotoxic T lymphocyte (CTL) response to H-2-compatible cells *in vitro*.

A question of major importance is whether there is a common genetic basis for these B and T lymphocyte abnormalitites. This question was approached by studying progeny of crosses between NZB and B10.D2 mice before three months of age for (1) altered expression of cell surface IgD, (2) splenic plasma cell frequency, (3) rates of IgM synthesis/plasma cell, (4) generation of a primary *in vitro* CTL response to BALB/c cells and, (5) production of antibody to T cells (TBA). At 10-11 months of age, the same animals were tested for circulating antibodies to red cells and thymocyte binding antibodies. The results showed that in this cross, alterations of cell surface IgD expression and production of red-cell specific antibodies are recessive traits, whereas plasma cell frequency, control of IgM secretion, production of TBA and the generation of CTL responses to BALB/c cells are governed by one or more dominant or semi-dominant genes. Specifically, plasma cell frequency appeared to be controlled by a single semi-dominant gene, IgM synthesis rate by one or two dominant genes, TBA production by 3 or more independently segregating dominant genes and the CTL abnormality by two genes at least one of which is semi-dominant. The data also suggested that there may be a single locus common to the genes regulating B cell defects.

Backcross mice were also analyzed by sepregation of alleles at Ly-9, Qa-1, Pep-3 and Gpd-1. Comparisons of CTL activity with these markers in backcross mice showed a significant correlation bewteen the ability to generate a strong anti-BALB/c CTL response and alleles at Ly-9. Thus, one of the two genes governing this response maps to chromosomal in linkage with Ly-9. Neither of the NZB CTL genes was associated with the NZB gene(s) governing polyclonal activation of B lymphocytes. (Drs. Davidson, Morse, Mathieson, and Chused).

GENETIC STUDY OF LEUKEMIA VIRUSES

The H-2 region on chromosome 17 of the mouse contains a series of genes which have been shown to play major roles in both cellular and humoral immune responses. A number of the loci in this region are highly polymorphic, but the basis for this polymorphism has yet to be defined. One possibility, raised by Dr. George Snell, is that MHC polymorphism develops in species with a heavy load of endogenous viruses but not in species which have a low virus load and that the resulting polymorphism acts to prevent the "threat" to survival posed by these organisms.

The cellular DNA of all mice contains multiple complete and incomplete copies of genetic information for xenotropic MuLV (X-MuLV). Infectious viruses of this class are incapable of reinfecting mouse cells but following recombination with ecotropic MuLV, certain parts of their genome can be transferred to other mouse cells. A documented effect of this transfer is the development of thymic lymphomas. In this form, X-MuLV genomes may thus provide a threat to the species. Studies from this laboratory strongly suggest that a gene or genes within the H-2 complex act to control the expression of infectious X-MuLV. The evidence is based on studies of F/St mice which produce high levels of X-MuLV. F_1 hybrids of F/St with AKR produce little or no infectious virus. Evaluations of (AKR X F/St)F_1 X F/St backcross mice demonstrated the suppression of X-MuLV was associated with a single dominant gene from AKR which assorted in tight linkage with $H\text{-}2K^k$ of AKR. The observation that B10.F mice (congenic for the H-2 complex of F/St) produce high levels of X-MuLV provides further support for the localization of this gene in association with H-2.

These findings raise the possibility that the postulated role for H-2 in tumor surveillance may occur through unexpected mechanisms and that X-MuLV may have some role in the development of MHC polymorphisms. (Drs. Morse, Davidson, Yetter, Hartley (LVD) and Rowe (LVD)).

CELLS FROM PAITENTS WITH SLE EXAMINED BY FLOW MICROFLUOROMETRY

We have examined the ratio of T cell subsets in patients with systemic lupus erythematosus and controls using monoclonal antibodies specific for T helper and T suppressor cells. The range of this ratio is significantly broader in SLE patients than in controls. We were able to devise a clinical score which correlated strongly with the helper-suppressor ratio. Patients with a low ratio tend to have severe renal disease, thrombocytopenia and onset of SLE before 20 years of age. Those with a high ratio tend to have multisystem disease including lymphadenopathy but only rarely SLE renal disease. These results suggest a previously unappreciated heterogeneity among SLE patients. (Dr. Chused).

A REVISED SCHEME OF T LYMPHOCYTE DIFFERENTIATION

The demonstration that Lyt $1^+2,3^-$ thymocytes precedes cells of the Lyt $1,2,3^+$ phenotype in ontogeny has revised the previously accepted view that Lyt $1,2,3^+$ cells differentiated into Lyt 1^+ or Lyt $2,3^+$ cells. A likely possibility is that there are two lineages of T cell precursors which are separable both phenotypically and functionally from early in ontogeny. Recent experiments with fetal thymic transplants indicates the Lyt $1,2,3^+$ thymocytes are descendents of Lyt $1^+2,3^-$ precursors, entirely reversing the previously accepted scheme of T cell differentiation. The transplantation work suggests that the differentiation of a microenvironmental inductive site is required for the expression of Lyt2 and Lyt3 antigens on the developing T cells. (Dr. Mathieson, Ms. Drakeford, Ms. Sharrow, and Mr. Leiserson.)

B CELL HYBRIDOMAS

Several BALB/C B cell tumors, adopted to *in vitro* continuous culture *in vitro* were mutagenized and grown in high concentrations of 6 thioguanine. One line M12.4 is sensitive to aminopterin and resistant to thioguanine. This line was fused with activated B cells from spleens of C57BL/6 (B6) mice. The resultant hybrids express IgM and H-2^b from the B6 partner. Certain hybrids released significant amounts of IgM into the culture medium following stimulation with lipopolysaccharide. It is probable that each hybrid clone represents a different stage of B cell differentiation. (Drs. Hamano, Kim, and Asofsky.)

ADMINISTRATIVE

Fogarty Center visitors C. Kanellopoulos-Langevin, Y. Rosenberg, and J. Hiernaux, left for positions at University College (London), the National Institute for Medical Research (London), and the University of Brussels, respectively. Dr. G. Chaouat returned to his position in Paris.

Joining the Laboratory in 1981 were Drs. A. Paraf, a Guest Worker from France. J. Langhorne, a Visiting Fellow from Basel; P. Smith a Guest Worker, and C. Taylor, a Visiting Fellow.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00134-19 LMI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Control of Immunoglobulin Synthesis in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	R. Asofsky	Laboratory Chief	LMI/NIAID
OTHER:	K. J. Kim	Visiting Associate	LMI/NIAID
	T. Hamano	Visiting Fellow	LMI/NIAID
	G. Chaouat	Guest Worker	LMI/NIAID
	Y. Rosenberg	Visiting Associate	LMI/NIAID
	C. Bever	Research Associate	LMI/NIAID
	B. J. Fowlkes	Microbiologist	LMI/NIAID
	Ada Brooks	Bio Lab Technician	LMI/NIAID
	William Leiserson	Mathematician	LMI/NIAID
	S. O. Sharrow	Chemist	IB/NCI
	L. Fitzmorris	IPA	LI/NIAID
	R. Lieberman	Microbiologist	LI/NIAID

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LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Experimental Pathology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.6	4.5	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Treatment of a B cell lymphoma with E. coli LPS resulted in an increase in surface IgM (sIgM but not Ia^d nor $H\text{-}2^d$. Treatment with β interferon increased expression of Ia^d and $H\text{-}2^d$, but not sIgM.

A B cell lymphoma was drug marked and B cell hybridomas obtained. Some of the hybrids secrete IgM. Others express IgM and $H\text{-}2^b$ from the strain of mouse, C57BL/6 used for fusion.

PHS-6040
(Rev. 2-81)

Project Description:

Work in the past year was concentrated on (1) neoplastic B lymphocytes, established *in vitro*, (2) B cell hybridomas made by use of a drug-marked B lymphoma, (3) a study of the requirements for T cell help in normal lymphocytes stimulated to produce immunoglobulins, (4) the effect of B-cell depletion on the development of sarcomas in strain SJL mice, (5) the effect of such depletion in C57BL/6 mice in the development of a normal repertoire of affinities of anti-DNP antibodies, and (6) the development of an animal model of *myasthenia gravis* induced by D-penacillamine, which is thought to be mediated by auto-antibodies.

1. Neoplastic B Lymphocytes

(Drs. Kim, Chaouat, Asofsky, Mr. Leiserson, Ms. Sharrow)

(a) Activation by Mitogens and Lymphocyte "Factors"

We have in the past established and cloned six B cell lymphomas of BALB/c origin *in vitro*. One such clone, X16C8.5 was chosen for detailed study. LPS from *E. coli* caused an increase in density of surface IgM, but not IgD, $H-2^d$, nor Ia-7. There was not secretion of IgM. Supernatent medium from spleen cells of various strains of mice, stimulated with the lectin conconavalin A did not increase surface IgM nor IgD, but did markedly increase expression of $H-2^d$ and Ia7 (detected by A. TH anti A. TL serum followed by fluorescein labeled, affinity purified goat anti mouse IgG_2). This activation was contained in a 30,000 - 40,000 M. W. fraction from G-100 chromatography. Activation was *not* seen with IL-I nor IL-II. A preparation from mouse fibroblasts, enriched for β interferon caused activation. Active protein synthesis was required to observe any activation.

(b) Characterization of a Surface IgG in a B Cell Lymphoma

(Drs. Kim, Fitzmorris(LI) and Laskov (Jerusalem) Mr. Leiserson)

Two clones of a BALB/C B cell lymphoma A20.2 and A20.1.11 have been shown to strain with fluorescein labeled goat anti mouse IgG_2. There was very little staining with anti IgM and variable staining with anti IgD, work is in progress to demonstrate γchain synthesis and mRNA for γchains.

In both parts of project 1, staining and amounts of stain displayed were measured by flow microfluorometry.

2. Establishment of B Cell Hybridonias In Vitro

(Drs. Hamano, Kim, and Asofsky, Mr. Leiserson)

Somatic cell fusion between normal B cells and B lymphoma cells has not been reported yet. Such B cell hybridomas might be good models for the study of B cell function. Several B lymphoma lines were mutagenized with ethyl methanesulfonate. One cell line, M12.4 expresses an immunoglobulin of unknown type (i.e. it stains on the surface with fluoresceinated anti-kappa light chain, but not with Ia anti IgM, IgD or any isotype of IgG) Ia^d, and $H-2^d$. After mutagenesis, a subline, M12.4.1 was shown to be resistant to 6 thioguanine and sensitive to aminopterin. This subline is thought to be deficient in the enzyme HGPRT. Somatic hybrids between M12.4.1 and spleen cells from immunized C57BL/6N(B6) were made in the presence of dimethylsulfoxide and polyethylene glycol. Fusion efficiency was about 10% of that obtained with NS-1. The B cell hybridomas obtained expressed membrane IgM (absent from the parent cell line), $H-2^b$ from the B6 partner as well as $H-2^d$ and Ia^d. Surface antigens were demonstrated by flow microfluorometry. Certain of the hybrids could be stimulated by LPS from E. coli to release IgM into the culture medium; others could not. The results so far demonstrate successful fusion.The hybridomas may express some functions of normal B cells. Future analysis will be made of other markers from the B6 partner as well as antigen. Specific receptors when fusions are done with B cells from immunized mice.

3. T Cell Regulation of Immunoglobulin Isotype Expression

(Drs. Rosenberg and Asofsky, Ms. Lieberman (LI))

A characteristic of immune responses to T dependent antigens is the highly restricted expression of antibody heavy chin classes. In addition to specific antibodies there is usually a marked increase in total Ig - secreting cells, which requires the presence of antigen specific helper cells and which exhibits an isotype restriction, similar to specific antibody made. This system was analyzed by mixing immune T lymphocytes with normal B lymphocytes and antigen, and measuring the number of IgFC produced by the indirect plaque assay. Log IgFC titered against log T cell numbers with a slope of about 1 for IgM and of about 2 for IgG_1, IgG_2 and IgA. T cells from mice suppressed from birth with anti IgM produced a good IgM response, but only a weak one for other isotypes. Similarly T cells from immunized immunoglobulin congeneic C.B-20 and BAB14 mice induced B cells to high IgM production, but little IgG_1 or IgG_2 production. It is hypothesized that IgG and IgA synthesis require 2 helper cells, one antigen specific, the second recognizing heavy chain determinants on $\gamma 1$, $\gamma 2$ and αchains.

4. Effects of Treatment of Mice with Anti IgM from Birth

(Drs. Katz, Thorbecke (NYU) and Asofsky)

(a) Development of Reticulum Cell Sarcoma (RCS) in SJL Mice

SJL/J mice were treated from birth with anti IgM. At 10 months of age such mice had 0 incidence of RCS as opposed to 60% incidence in controls. Two established lines of RCS injected i.v. into anti IgM suppressed mice grew at half the rate they did in normal mice. T cell functions seemed normal in anti IgM treated mice.

(b) Development of an anti DNP repertoire. (Drs. Goidl and Siskind (Cornell Medical School) and Asofsky). C57BL/6 mice have only a narrow range of inducible anti DNP antibodies in the first two weeks of life. Afterwards a very broad range of anti DNP affinities are induced. Mice suppressed with anti IgM from birth show only a narrow range of affinities for 5 weeks after anti IgM treatment was stopped, and in spite of the fact that normal numbers of B cells were present. Future work will attempt to determine if this is an induced B cell deficiency, or a deficiency in helper T cells.

5. The Induction of Myasthenia Gravis by D-penicillamine

(Drs. Bever, Baker, Rollwagen, and Asofsky, Ms. Fowlkes, Dr. Penn and Chung (columbia))

Treatment with D-penicillamine (DP), a drug used widely in the treatment of rheumatoid arthritis, has been associated with a variety of autoimmune side effects including myasthenia gravis (MG). The mechanisms for the therapeutic and autoimmune side effects are unknown but there is evidence to support both an effect of DP on immunoregulation and a direct chemical effect on host proteins which could lead to a loss of tolerance. We have undertaken chronic treatment of inbred strains of mice (A/J, AKR, BALB/c, C3H, C57BL6/J, and DBA-1) for up to 6 months. None of the mice developed symptoms or signs of MG. Stored sera are now being screened for anti AchR antibody. Chronic treatment of BALB/c mice did not significantly effect surface phenotype of thymocytes or splenic lymphocytes, proliferative responses of splenic lymphocytes to Con A or LPS or number of plaque forming spleen cells to SRC's or SIII, but did lead to an enhanced anti AchR antibody response after challenge with purified AchR from T. Calif. The response to challenge with purified AchR is the established model of MG so we have been working to adapt it to the problem of DP induced MG. We have demonstrated that DP with chemically alter purified AchR and are studying anti-AchR antibody responses to native and DP treated AchR.

We have been unable to show any difference using AchR from T. Calif., which is itself highly immunogenic, so we are currently preparing AchR from mouse muscle to use in similar studies.

Publications

Katz, I. R., R. Asofsky, and G. J. Thorbecke. 1980. Suppression of spontaneous reticulum cell sarcoma development and of syngeneic stimulator cells by anti-μ treatment of SJL/J mice. J. Immunol. 125:1355-1359.

Kim, K. J., C. Kanellopoulos-Langevin, G. Chaouat, and R. Asofsky. 1981. Differential effects of mitogen and nonspecific T cell factors in the expression of surface immunoglobulin (sIg) and Ia antigens on B lymphocyte cell lines. In, B Lymphocytes in the Immune Response, M. Cooper, D. Mosier, I. Scher and E. S. Vitetta, (Eds.), Elsevier North-Holland, New York, pp 507-514.

Rosenberg, Y. J., R. Lieberman, and R. Asofsky. 1981. The production of IgG and IgA requires signals from a second population of helper cells specific for IgH-C or closely linked determinants. Proceedings of the Second International Conference on B Lymphocytes. Scottsdale, Arizona, October 1980.

Rosenberg, Y. J., R. Lieberman, and R. Asofsky. 1981. T cell regulation of isotype expression. The requirement for a second Ig-specific helper T cell population for the induction of IgG responses. Eur. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00135-07 LMI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Properties of Immunoglobulin Secreting Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during current year.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00136-09 LMI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Functional Activities of Subpopulations of Thymus-Derived Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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2.9	1.9	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Examination of Lyt differentiation markers on thymocytes during murine ontogeny has led to a revised scheme of thymocyte differentiation. This scheme proposes 2 independent precommitted lineages of thymic precursor cells and a site-specific inductive signal for functional and phenotypic differentiation.

Fetal thymus transplantation data supported this concept and transplants into chimeras and nude mice demonstrated a disparity between the genetic expression of Lyt markers that are associated with functional subsets of interactive cells and the MHC markers, which on thymoytes, appeared to be expressed according to their thymic environment.

Positive selection for Lyt 2 antigen has allowed the separation of the functional lineages for helper T cells, cytotoxic T cells and synergistic interactive T cells and thymus subsets involved in mixed lymphocyte and graft versus host reactions.

PHS-6040
(Rev. 2-81)

1. Expression of T-cell Differentiation Cell Surface Antigens on Thymocytes and Thymus-Derived Cells.

Flow microfluorometry analysis of thymocyte subpopulations has been extended for Lyt antigens and other cell surface differentiation markers (See previous project report for FY 1980). The major focus of this research has been to analyze the question of 2 separate lineages of thymocytes and thymus-derived cells, both by phenotype and by function. This now has been demonstrated from early stages of fetal thymic development and we now are pursuing the question of whether there are 2 independent pre-committed thymic lineages which can be detected in adult bone marrow and fetal liver.

A. Fetal Thymocyte Differentiation: Most thymocytes are either immature or functionally deficient and express a series of lymphocyte cell surface antigenic markers designated Lyt1, Lyt2, and Lyt3, that have been useful in distinguishing functional subsets of T cells. In contrast, a small population of cortisone-resistant thymocytes (CRT), confined largely to the thymic medulla after acute corticosteroid treatment are functionally more mature. Some of these cells, like peripheral T cells, have restricted expression of Ly antigens and appear as either Lyt1$^+$2,3$^-$ or Lyt1$^-$2,3$^+$ cells. It thus has been assumed that all thymocytes are Lyt1,2,3$^+$ and that during differentiation lose either Lyt1 or Lyt2,3 to result in Lyt1$^+$,2,3$^-$ and Lyt1$^-$2,3$^+$ cells.

Using immunofluorescence (IF) and flow microfluorometry (FMF) analyses to detect Lyt antigen expression quantitatively without the requirement for cell lysis, we now have re-examined the expression of Lyt1, Lyt2, and Lyt3 antigens on murine fetal thymocytes from 14 to 19 days of gestation and on normal thymocytes from birth to 2-3 month old adults. These studies demonstrate that cells with the Lyt1$^+$2,3$^-$ phenotype first appear in the thymus several days before Lyt1,2,3$^+$ thymocytes are detected. Thus what was presumed to be a more differentiated phenotype is observed at 14 to 16 days gestation before the first Lyt1,2,3$^+$ cells are observed at day 16 to 17. This work suggests either a microenvironmental or site-specific influence for phenotypic differentiation and/or two independent, precommitted lineages. (Mathieson et al., Nature 1981).

B. Analysis of Fetal Thymocyte Differentiation in Thymic Grafts. The data that we obtained on fetal thymocyte differentiation raised the issue of whether Lyt1$^+$2,3$^-$ thymocytes in the fetal thymus are differentiating into Lyt1,2,3$^+$ cells or whether a second lineage of incoming cells accounted for the apparent Lyt1,2,3$^+$ differentiation in the phenotype of the fetal thymocytes on day 17. To address this issue, a series of fetal thymus transplants were performed. In these experiments, fetal thymi from embryos at 15 days gestation, when only Lyt1$^+$2,3$^-$ cells are observed, were transplanted under the renal capsule of congenic mice with Lyt1,2,3 allelic differences. Cells from the transplants were monitored for both allelic forms of the Lyt antigens at 2, 5, 15, and 30 days after transplantation.

When cells from the thymic transplants were examined at 2 days after transplantation, 60 to 70% of the cells were $Lyt1,2,3^+$, i. e. at the chronological age of day 17 gestation for the thymus graft the cells expressed the phenotype that they would have had in situ. By 5 days, the normal newborn and adult phenotype (10% $Lyt1^+2,3^-$ and 90% $Lyt\ 1,2,3^+$) was observed on the cells from the thymic grafts. Furthermore from 2 days to 15 days after grafting, the allelic forms of the Lyt antigens expressed on the thymocytes indicated that cells already in the graft at day 15 gestation were differentiated into $Lyt1,2,3^+$ cells.

Thus these analyses give clear evidence of maturation of $Lyt1,2,3^+$ cells at the point when the fetal graft stroma reaches the 17 day age point. This indicates the possibility of a site-specific differentiation for the $Lyt1,2,3^+$ lymphoid cells that is dependent on the differentiation of the thymic epithelial microenvironment. Since $Lyt1^+2,3^-$ cells continue to be detected throughout this period in a relatively constant number, this further supports the concept of 2 independent lineages of thymocytes. (Mathieson and Sharrow, presented at the Dec. 1980 meeting of the American Society of Zoologists in Seattle, WA; manuscript in preparation.)

Further analysis of the functional differentiation of the fetal thymocytes during the critical time point of differentiation of Lyt antigens is in progress. In particular we are investigating the time at which thymocytes become functionally competent for cytotoxic function both for allogeneic and altered self responses. We also are investigating the capacity of 15 day fetal thymocytes deprived of the in situ environment for further differentiation as well as their ability to produce T cell factors such as interleukin 2 (IL 2).

C. Analysis of Irradiation, Bone Marrow Chimeras and Thymic Transplants. Studies of thymocytes from bone marrow repopulated, irradiation chimeras have analyzed the cell surface phenotype with regard to the expression of major histocompatibility (MHC) antigens as well as Ly antigens (Sharrow, Mathieson, and Singer. J. Immunol. 1981). These studies have demonstrated acquisition of host MHC antigenic phenotype on intra-thymic cells, genetically identified as donor cells by Ly antigen expression.

Corollary experiments demonstrate that there is initially a total repopulation of the thymus with a radioresistant intrathymic stem cell(s). In relation to the sections above, this initial thymic repopulation with its subsequent donor replacement appears to support the possibility of site-specific differentiation but does not eliminate separate precommitted lineages which have different intrathymic homing patterns.

Functional analysis of thymic cells in donor reconstituted mice (Kruisbeek, Sharrow, Mathieson, and Singer, 1981, manuscript submitted for review) indicates MHC associated restriction correlated with the thymus graft type in either chimeras or grafts. This further demonstrates the disparity between the genetic identification by Ly phenotype and the MHC antigen phenotype; the latter appears to be non-genetic for thymocytes.

D. Analysis of Thymic and Prethymic Cell Lineages. The major area of my current research involves the question of whether there are two lineages of prethymic cells. We have pursued this possibility because a number of studies have indicated heterogeneity in the lymphoid precursors from the bone marrow. Ly9 (Lgp 100), a lymphocyte antigen which we and others have described recently (Mathieson, et al., J. Immunol. 1980), appears, by analysis of FACS sorted cells, to detect two subsets separable by size in the bone marrow. Thus, with antigenic phenotype and cell size, we have the tools to separate and analyze the question of two precommitted thymic lineages. If such separation is possible, the potential for identification of the precursors of cells active in graft-versus-host (GVH) disease then may be accomplished. Use of fractions of bone marrow then will allow us to analyze the development of chronic GVH reactions.

2. Characterization of Lectins as Novel Markers on Murine and Human lymphocytes

As an alternative to antibody probes to cell surface antigens, we are continuing to examine by FMF a number of plant and animal lectins capable of recognizing complex carbohydrate moieties on glycoprotein or glycolipid structures of the cell surface. Following our successful application of peanut lectin (PNA) fractionation to enrich minor subpopulations of thymocytes, we have expanded these studies to a panel of about 50 other lectins. We have further identified and subdivided thymocyte subpopulations, for which there is additional evidence by physical fractionation and cell surface antigenic characterization for functional heterogeneity.

These studies largely have involved a body of work which is a collaborative effort between B. J. Fowlkes, (LMI), Dr. B. J. Mathieson (LMI), Dr. M. J. Waxdal (LI), Charles Thomas, LMI/LCI), Dr. Barton Haynes (LCI, currently at Duke University, NC), S. O. Sharrow (IB,NCI) and Dr. Marion Zatz (George Washington Medical Center, DC). These and several other colleagues have participated in the analysis of this panel of lectins binding to subpopulations of cells. We now have completed survey work on various lymphoid populations from mice and on peripheral blood lymphocytes and spleen cells from humans.

A. Characterization of Lectin Markers on Thymocytes. Initially we demonstrated that a number of fluoresceinated lectins bind differentially to thymocyte subsets as detected by FMF (Fowlkes et al., J. Immunol. 1980). The lectins that are of primary interest in these studies are series of plant lectins with nominal binding specificity for galactose, N-acetyl-galactosamine or polymers of N-acetyl-glucosamine. A number of fluorescein-labelled lectins (Fl-lectin), have shown bimodal or trimodal fluorescent profiles on thymocytes. For many of these lectins we have shown that the dull fluorescent subpopulation is enriched in the CRT fraction, in parallel with an enrichment for Thy-1 dull and Lyt1$^+$2,3$^-$ thymocytes. Using peanut lectin to fractionate thymocytes we have further been able to enrich and recover both the dull population of many of these other lectins and the bright staining population that is lost in cortisone treated mice.

We have continued to analyze the relationships between the carbohydrate binding sites of the lectins and the anti-Ly sera which we currently have available. In this regard, abnormal dystrophic (*dy*) mutant mice appear to have a pattern of thymocyte phenotype anomalies in some of the affected mice. These mice appear to have a higher proportion of CRT-like cells by phenotypic analysis with antisera to thymic antigens and a series of lectins including PNA (Ludwig et al., *Cell. Immunol.* 1981).

Additional work by Chaouat, Fowlkes, Leiserson and Asofsky, (manuscript in preparation) has further demonstrated the usefulness of the lectin markers for analysis of thymic subpopulations. In this work, shifts in the thymic subsets in allopregnant versus syngeneically pregnant mice have been observed. Modifications of thymus subsets during pregnancy were monitored using FMF and Fl-lectins or antibodies. The percentage of PNA$^-$ cells increased significantly during pregnancy. More interestingly, in the strain tested, the percentage of PNA$^-$ cells was more highly increased in allogeneic pregnancy than in the syngeneic pregnancies. These increases in PNA$^-$ cells could be mimicked by extracts from syngeneic placenta, but the largest increase in PNA$^-$ cells always was observed by injection of allogeneic placental extract, or syngeneic placental extract plus allogeneic cells. These findings suggest that the alloantigenic status of the conceptus does play a role in the induction or maintainance of thymus involution. This in turn suggests that the altered phenotype is not a mere hormonal phenomenon and that shifts in the thymic subpopulations could reflect the events leading to the acceptance of the allogeneic conceptus by the maternal immune system. These shifts in subpopulations presumably reflect an immunogenic stimulus in the animals and the driving force for differentiation and peripheralization of thymus-derived cells.

B. Characterization of Lectin Markers on Splenic Lymphocytes

The binding of fluorescein-labeled lectins bound to mouse spleen cells has been analyzed by flow microfluorometry (FMF). This screening has identified several lectins which bind preferentially to sIg+ spleen cells. There was little correlation seen between the type of fluorescence distribution obtained and the nominal carbohydrate specificity of lectin. Lymphocyte subpopulations delineated by FMF analysis of lectin binding were further characterized on spleen cells separated with anti immunoglobulin coated plates. Lymphocyte were characterized also by two-color FMF of cells doubly stained with lectin and goat anti-mouse kappa or monoclonal anti-Thy1.2. These studies indicated that a number of lectins bind preferentially to sIg+ spleen cells particularly SBA, WGA, Pa-2, and Spud some of these lectins also detected subsets within the sIg+ spleen population.

Because of lectin markers have proven useful to detect subpopulations in other mammalian species we have extended these studies to the analysis of human lymphocyte and monocyte subpopulations. These several aspects are being investiaged in collaboration with Dr. M. J. Waxdal (See project Z01-A1-00038-09 LI) and Barton Haynes, LCI.

3. Analysis of the Expression of Lymphoid Differentiation Antigens by Trypsinization and Regeneration.

Using flow microfluorometry to detect immunofluorescence, the expression of Lyt 2, Lyt 3 and Ly 9 antigens on thymocytes and regeneration of these antigens after trypsinization was studies. These analyses have demonstrated that Lyt 2.1, Lyt 2.2, Lyt 3.2 and Ly 9 antigens are removed easily from the cell surface by trypsinization. In contrast, Lyt 3.1 is partially resistant to trypsinization on the cell surface using the same conditions under which Lyt 2.1 is sensitive. Thus, Lyt 3.1 is not expressed on the same polypeptide molecule as Lyt 2.1 or it is not equally accessible or sensitive to tryptic cleavage on the unmodified viable cell surface. The trypsin resistance of Lyt 3.1 also was found to be independent of association with either allele of Ly 9. The resistance of Lyt 3.1 to tryptic cleavage was found consistently on thymocytes of several Lyt 3.1 strains in contrast to the sensitivity of Lyt 3.2 on thymocytes from strains expressing the opposite allele, thus it appears to be allele specific.

The regeneration of Lyt 3.2 or either allele of Lyt 2 to normal levels of antigen expression on thymocytes requires 24 hr. The first reexpression of these antigens can be detected at the cell surface at 3 hr. In contrast, only about half of the normal Ly 9 antigen level can be found even at 24 hr, and indicated independent expression of these antigens. These studies demonstrate that the Ly antigens described are, in fact, of thymocyte-T cell origin. In these experiments a separate and distinct subpopulation (10%) expression the phenotype Lyt 1+2, 3- consistently was identified in contrast to the majority of normal thymocytes which are Lyt 1, 2, 3+. (Robert Hooghe, Bonnie J. Mathieson, Susan O. Sharrow, Stuart Rudikoff, and K. Jin Kim. Differential Trypsin Sensitivity and Reexpression of Ly Antigens on Thymocytes. Manscript submitted for review.

Additional experiments with enzymatic digestions of cell surface markers may prove useful for analysis of the relationships of surface antigen markers to functional capacity of the cells.

4. Analysis of the Genetics of Cell Surface Markers

This work encompasses four individual projects which we recently have completed or which are currently being completed, (a) The genetic linkage of Lyt2,3 T cell antigens with kappa light chain markers has intriguing implications for T cell regulation associated with selected representation of light chains in the immune response to immunogens such as PC. We initially investigated this association in the NZB X C58 (NX8) recombinant inbred strains of mice in collaboration with Dr. Roy Riblet at Fox Chase Cancer Center, Philadelphia (Riblet et al J. Immunol. 1980). Further analysis of the NX8 mice now has indicated a recombination event between Ig kappa markers and the Lyt2,3 genotype. (b) We also have pursued the association of Lyt2,3 by the analysis of families of genetically recombinant mice and congenics produced by Dr. David Gibson, Canada and Dr. Marianna Cherry (Jackson Laboratory, Bar Horbor, Maine). In these studies we have demonstrated the dissociation of the Lyt markers with electrophorectic Ig kappa markers which Dr. Gibson has investigated more extensively. Thus these two studies predict an independent relationship of the Lyt2,3 and Ig kappa markers. However, the recombinants which Dr. Gibson has observed are all loss variants and the reciprocal phenotype has not been observed. (c) In both of the above studies, the mice were monitored for a second locus associated with the Ly 9 antigen described above in section 1D. in Addition the collaboration with Drs. H.C. Morse III, Wendy Davidson C. Kozak, J. Crowle and R. Lieberman, formal backcross analysis and studies of RI strains has mapped Ly 9 to chromosome 1 with no recombinants between Ly9.1 and Lgp 100 an antigen described by Jeff Ledbetter at Stanford. Further collaborative genetic analyses with Ly 9 linked antigens has demonstrated a complex of cell surface interaction molecules which are associated with fraft rejection, mixed lymphocyte interaction, B cell stimulation and xenotropic murine leukemia virus induction. (See Z01 A1 00205-01 LMI). In addition, a survey of wild mouse strains now has been completed with Drs. Isabella Pawlita and Michael Potter. This survey, in addition to yielding information about the phylogenetic relationship of these mice, has shown an apparent dissociation for Ly9.1 and Lgp 100 which was not demonstrable in conventional inbred strains of mice.

5. Characterization of Thymocytes and T Cells Grown in Interleukins

We have analyzed the phenotype of cells stimulated in vitro with interleukins IL1 (LAF) and IL2 (T cell growth factor; TCGF). We have examined the T cells derived from normal spleens and from T cells infiltrating syngeneic solid tumors which were expanded for 1 to 3 weeks in TCGF and we have examined the short term phenotypic alterations on thymocytes observed up to 72 hrs with interleukins with or without mitogen stimulation.

A. Normal murine splenocytes and tumor derived lymphocytes grown in bulk cultures in TCGF are lytic for fresh or cultured syngeneic tumor cells and a variety of cultured murine cells. To further characterize the cells responsible for this lysis we studied their cell surface markers. We have demonstrated that splenocytes grown in TCGF for 1 week express Thy-1, Lyt-2 and low levels of Lyt-1. When cells are maintained in bulk cultures for more than 2 weeks Lyt-1 is no longer expressed on the cell population and the cells uniformly express the conventional CTL phenotype which is Thy-1+ Lyt (1-) Lyt-2+. The phenotypic expression of these cell surface markers was analyzed by FMF for quantitative determine the phenotype, independent of a requirement for complement dependent lysis. Lysis experiments were conducted in parallel to analyze the effect on CTL function.

The uniformity of the phenotype is not due to antigen shedding and pick-up because our analysis of co-cultures of cells expressing the two alternative alleles of the same antigen indicates separate definitive populations for both alleles of Thy-1 and of Lyt-2. Tumor derived lymphocytes grown in TCGF also expressed these same CTL surface markers. Our experiments with congenic mice show that the T cells growing in TCGF originated from the host T-cells residing in the tumor.

B. We are currently analyzing the interactive capacity of fractionated subsets of thymocytes with mitogen and factor stimulation on collaboration with Drs. Michael Mage and Joost Oppenheim. These studies have indicated both qualitiative and quantitative differences in the types of signals the cells receive for proliferation from these stimuli.

6. Functional Analysis of Thymocyte and T Cell Subsets

Our current efforts in this area are related to two aspects of functional analysis of thymocyte subsets. This involves the analysis of positively selected Lyt 2+ and Lyt 2- subsets of PNA fractionated cells from synergy in GVH with Dr. Michael Mage, NCI. These studies follow work recently published (Mage et al., Eur. J. Immunol. 1981) and previous studies (Betel, et al., J. Immunol. 1980 and Mathieson, et al., Protides Meeting, 1979) which demonstrated Lyt subsets within PNA fractionated thymocytes.

A. Positive Selection of Functional Subsets of Lymphocytes. A convenient, preparative scale, non-lytic separation of mouse T lymphocytes into Lyt2.2$^+$ and Lyt2.2$^-$ populations was developed in collaboration with Dr. Michael Mage, NCI. Ig$^-$ spleen cells, Ig$^-$ lymph node cells, and peanut lectin-unagglutinated (PNA$^-$) thymocytes were incubated under sterile conditions at 0°C with monoclonal mouse antibody to the Lyt2.2 T cell differentiation antigen. The antibody-treated cells were washed and placed in polystyrene tissue culture dishes that had been precoated with antibody to mouse immunoglobulin. Nonadherent populations were depleted of Lyt2.2$^+$ cells and were essentially devoid of cytotoxic T lymphocyte precusors (CTLp), but contained helper activity for in vivo T-dependent IgM, IgG, and IgA antibody formation. Adherent cell populations were enriched for Lyt2.2+ cells and for CTLp. The GVH activity of the separated adherent (Lyt2.2$^+$) and non-adherent (Lyt2.2$^-$) cells in the Simonsen spleen assay in neonatal (C57BL/6 x BALB/c)F$_1$ mice was less than that of unfractionated cells, but the activity of re-mixed Lyt2.2$^+$ plus Lyt2.2$^-$ cells was higher than the sum of the contributions of these cells tested separately, and equal to that of this unfractionated cells. PNA$^-$ thymocytes were also separated into Lyt2.$^+$ and Lyt2.2$^-$ populations by fluorescence activated cell sorting. Nonlytic separation allows the recovery of the Lyt1$^+$2$^+$ population, which is lost in cytotoxic elimination experiments. Under this conditions described for the plate separation, the purity of the separated cells and recovery of activity approaches that of cells separated by sorting. Therefore, the plate separation offers a convenient alternative to fluorescence activated cell sorting when large numbers (i.e. up to 5 x 10^7 positively selected cells) are needed, as in studies of in vivo cell mediated immune reactions.

B. Evidence for the Requirement of Two T Cells to Interact in the Generation of Effective Suppression. The cellular requirements for suppression of a mixed lymphocyte reaction (MLR) by *in vivo* alloantigen-activated spleen cells were investigated. It was found that the suppression was mitomycin resistant at days 3 and 5 after antigen injection. By contrast, it was radiation sensitive on day 3 but not on day 5. Cell fractionation studies using a plate separation technique monitored by flow microfluorometry were performed. It was found that neither purified Lyt2$^-$ cells nor purified Lyt2$^+$ cells independently could suppress an MLR. By contrast, the reconstituted T population was still suppressive. It also was found that culture supernantant factors from allogeneically *in vivo* restimulated Lyt2$^-$ cells could suppress and MLR if Lyt2$^+$ cells were present in the responding population. These data obtained by positive selection and reconstitution show that at least 2 surface immunoglobulin-negative cells interact for the generation of effective MLR suppression. The possibility that we are dealing with a suppressor-inducer, suppressor-effector system is discussed.

7. Thymocyte Responses to Antigen Stimulation

Thymocytes (ThC) and lymph node cells (LNC) interact synergistically to provide greater than additive responses to proliferative and cytotoxic reactions to alloantigen. Last year, we reported that injection of antigen (SRBC, OVA) resulted in increased capacity of ThC to respond to T-cell mitogens and to alloantigens. We have extended these findings to show that such synergistic interactions occur in the proliferative response to soluble (OVA), but not to particulate (SRBC) antigens. LNC must be primed to the appropriate antigen, but ThC must be supplied by naive mice. We interpret these results to suggest that a nonspecific signal is provided by the thymocytes which enables the LNC to undergo greater specific proliferation. In addition, ThC from mice injected with OVA are able to suppress specifically the proliferative response of primed LNC to OVA *in vitro*, but did not proliferate themselves in response to antigen. In parallel experiments investigating responses to particulate antigens (SRBC), we found that no such suppressive mechanism existed. These findings suggest that the thymus is not protected from antigenic stimulation, and responds to antigen by generating a population(s) which is Thy1.2 "dull" and responsive to mitogens and alloantigens, but which is specifically suppressive to the inducing antigen. Further experiments to clone and/or hybridize this thymic cell subpopulation are underway.

Publications

Mathieson, B. J., S. O. Sharrow, K. Bottomly and B. J. Fowlkes. 1980. Ly9, an alloantigenic marker of lymphocyte differentiation. J. Immunol. 125:2127-2136.

Mathieson, B. J., S. O. Sharrow, Y. Rosenberg and U. Hammerling. 1981. Lyt 1$^+$23$^-$ cells appear in the thymus before Lyt 123$^+$ cells. Nature 289:179-181.

Zatz, M. M., B. J. Mathieson, C. Kanellopoulos-Langevin, and S. O. Sharrow. 1981. Separation and characterization of two component tumor lines within the AKR lymphoma, AKTB-1, by fluorescence activated cell sorting and flow microfluorometry analysis. I. The coexistence of sIg^+ and sIg^- sublines. J. Immunol. 126:608-613.

Sharrow, S. O., B. J. Mathieson, and A. Singer. 1981. Cell surface appearance on unexpected host MHC determinants on thymocytes from radiation bone marrow chimeras. J. Immunol. 126:1327-1335.

Ludwig, C. L., C. Kanellopoulos-Langevin, K. J. Kim, B. J. Mathieson, and H. C. Morse III. 1981. Immunologic function and cell surface antigen expression of lymphocytes of dystrophic mice. Cell. Immunol. 59:138-150.

Mage, M., B. J. Mathieson, S. O. Sharrow, L. McHugh, U. Hammerling, C. Kanellopoulos-Langevin, D. Brideau, Jr., and C. A. Thomas III. 1981. Preparative non-lytic separation of Lyt 2^+ and Lyt 2^- T lymphocytes and demonstration of synergy in graft-versus-host reactions of Lyt 2.2^+ and Lyt 2.2^- lymphocytes. Eur. J. Immunol. 11:228-235.

Oppenheim, J. J., B. Stadler, R. Siraganian, M. Mage and B. J. Mathieson. 1981. The properties of Interleukin 1 (IL1) and generation of monoclonal anti-IL1. Federation Proceedings, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00137-15 LMI

PERIOD COVERED
October 1. 1980 to September 30. 1981

TITLE OF PROJECT (80 characters or less)

Biology of Graft-Versus-Host Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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0.7	0.7	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Thymocytes taken from mice undergoing a GVHR show phenotypic differences relative to normal mice, as analyzed by flow microfluorometry. They also show increased Con A and MLR responses. These changes are the result of antigen recognition by either the host or the grafted cells.

Project Description:

ANALYSIS OF T AND B CELL FUNCTIONS AND PHENOTYPES IN MICE UNDERGOING A GRAFT-VERSUS-HOST REACTION (GVHR)

Adult C3H x DBA/2 F_1 mice injected with viable spleen cells from one parental strain undergo a graft-versus-host reaction (GVHR) characterized by the development of splenomegaly, increased prostaglandin production, and immunosuppression. We have repeated these findings, and show a 2-3 fold reduction in Con A responses by spleen or lymph node cells taken from mice undergoing a GVHR. Flow microfluorometry analysis of spleen cells taken from such mice reveals an increase in surface immunoglobulin expression. Expression of Thyl on these spleen cells, however, was reduced relative to uninjected mice.

We have shown that thymocytes (ThC) taken from mice undergoing a GVHR show increased Con A and MLR responses, as well as an increase in Thy 1.2 "dull" cells. These findings are consistent with our previous report that various antigens induce functional and phenotypic changes in thymocytes (see report 000136-09). Binding of the plant lectins, phytohemagglutinin (PHA) and *Abrus precatorius* (APA) was decreased on ThC taken from mice undergoing a GVHR. These lectins bind the sugars N-Acetylglactosamine and β-galactose, respectively, on the cell surface. The β-galactose binding lectin derived from *Malcura pomifera* (MPL), however, was shifted brighter on such ThC. We are currently investigating functional subpopulations binding such lectins.

It is apparent that recognition of antigen by peripheral lymphocytes induces a systemic response (possibly via factors) which results in thymic changes. It is not yet clear whether these changes are the result of antigen recognition by the host or by the graft. Further experiments are underway, using millipore chambers and cell transfers which may serve to answer some of these questions.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00138-07 LMI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Viruses and the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	R. A. Yetter	Guest Researcher (IPA)	LMI/NIAID
	C. A. Kozak	Staff Fellow	LVD/NIAID
	W. F. Davidson	Expert/Consultant	LMI/NIAID
	P. M. Hoffman*		
	B. A. Taylor**		
	J. B. Roths**		
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2.9	1.9	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A high proportion of thymocytes from F/St mice produce xenotropic murine leukemia viruses (X-MuLV) but expression of infectious virus is markedly suppressed in all F_1 hybrids of F/St with other strains. Suppression of X-MuLV is governed by a single dominant gene which maps within or very close to the H-2 complex. This gene appears to exert a negative regulatory influence on a chromosome 1 X-MuLV induction locus.

NFS mice infected with a wild mouse ecotropic MuLV develop splenic lymphomas and hind limb paralysis. Dualtropic MuLV appear in spleen cells but not thymocytes or brain prior to the onset of either disease. The lymphomas appearing in these mice are predominantly erythroleukemias and their development is accelerated by treatment with antithymocyte serum.

NFS mice congenic for the AKR ecotropic MuLV loci develop splenic lymphomas between one and two years of age. These tumors, previously classified as reticulum cell sarcoma Type B, have been shown to be neoplasms of B lymphocytes.

A dominant gene for resistance of mouse tissues to infection with dualtropic MuLV was mapped to chromosome 5 in close linkage with Hm.

Project Description:

These studies reflect long standing collaborations with Dr. Janet Hartley of the Laboratory of Viral Diseases on the expression of MuLV in lymphoid tissues.

Expression of infectious X-MuLV. F/St mice produce high levels of infectious X-MuLV and ecotropic MuLV. The high ecotropic virus phenotype was found to be governed by multiple (4 or more) independently assorting dominant loci. The high X-MuLV phenotype was shown to behave as a recessive trait in all F_1 hybrids tested. In (AKR x F/St)F_1 x F/St mice it was found that a single dominant gene linked to *H-2*K^k effected suppression of X-MuLV. The suggestion that the *H-2* complex of F/St was permissive of X-MuLV expression was confirmed by the observation that the *H-2* congenic strain B10.F/Sg also produced high levels of X-MuLV.

Mice carrying the mutant gene *lpr* develop massive lympho proliferation and severe autoimmune disease. SJL mice which are *lpr/+* develop peripheral lymphomas at an accelerated rate as compared to *+/+* mice. To determine if the *lpr* gene might alter virus expression (as a possible basis for lymphoma development) SJL, NZB, C3H, AKR, and C57BL/6 mice carrying the *lpr* gene were tested for expression of ecotropic and X-MuLV in lymphocytes. The *lpr* gene was found to activate ecotropic MuLV in SJL mice but not the other strains tested whereas activation of X-MuLV was detected in NZB mice but not the other strains including SJL. Studies are in progress to determine how activation of these MuLV loci might affect the strains carrying this mutation.

Studies of wild mouse ecotropic virus. NFS mice exposed to CAS-BR-M virus before 10 days of age develop hind-limb paralysis (the pathology of which resembles amyotrophic lateral scerosis) and lymphomas. NFS mice older than 10 days do not develop either disease following virus inoculation and other strains, such as DBA/2, have a much delayed onset of disease following neonatal exposure to virus. Our studies have been directed at pathogenesis of these distinct diseases caused by a single agent. It was found that spleen cells, but not thymocytes or brains of CAS-BR-M infected mice, produce dualtropic (MCF) MuLV soon after inoculation. The virus is expressed in non-T, non-B splenocytes which may be the targets for neoplastic differentiation in that many of the tumors which subsequently develop appear to be erythroleukemias. MCF MuLV may ths be involved in the development of lymphomas but not paralysis. In addition, inoculation of mice treated with anti-lymphocyte serum accelerated the development of lymphomas but does not appear to influence the development of neurologic disease.

Studies of Akv-congenic mice. NFS mice congenic for the AKR ecotropic MuLV loci experience a high incidence of non-thymic lymphomas. By histologic criteria, the tumors had been classified as reticulum cell sarcomas Type B. These tumors were analyzed with a variety of antibodies specific for markers on T or B lymphocytes and the great majority of them were found to have characteristics of B cells. A review of the histology indicated that the tumors could be reclassified in accordance with the Lukes-Butler nomenclature for human B cell tumors into plasmacytoid, small cleaved, large and immunoblastic types. Akv-congenic mice thus provide a model system for study and treatment of B cell lymphomas.

Resistance to MCF MuLV. A single dominant gene in DBA/1, DBA/2, and CBA/Ca mice determines resistance to infections of mouse cells with MCF MuLV. Linkage analyses using AKR (MCF sensitive) by DBA/2 recombinant inbred strains and backcross mice demonstrated that this gene is linked to Hm on chromosome 5. An AKR mouse congenic for this locus is being developed.

Publications:

Hoffman, P. M., S. K. Ruscetti, and H. C. Morse III. Pathogenesis of paralysis and lymphoma associated with a wild mouse retrovirus infection. Neuroimmunology, 1981, in press.

Hoffman, P. M., W. F. Davidson, S. K. Ruscetti, T. M. Chused, and H. C. Morse III. Wild mouse ecotropic murine leukemia virus infection of inbred mice: dual-tropic virus expression proceeds the onset of paralysis and lymphoma. J. Virology, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00139-16 LMI

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Immunologic Response of Animals to Trypanosomal Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	P. Gardiner	Visiting Fellow	LPD/NIAID
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	J. Jones**	Assistant Professor	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mice infected with various species of African trypanosomes, succumb 23-120 days after infection. Survival depends on the presence of IgM antibody. Mice with delayed IgM responses survive the longest. Low Clq binding correlated with long survival time.

PHS-6040
(Rev. 2-81)

Project Description:

African trypanasomiasis is an infection of the blood compartment of mammals, characterized by successive wave of antigenic variants in the parasite population. Studies of the Ig isotype response, has been limited to IgM assays. Our studies focused on the specific isotype response to 2 trypanasomes: *Trypanosoma brucei brucei*, an animal pathogen, and *T. b. rhodesiense*, a human parasite. Both of these parasites readily infected laboratory animals. The use of both parasites was to ensure that the response was a characteristic of the host rather than the parasites strain *per se*.

CBA/N, CBA/CAJ, and nude mice were infected with *T. rhodesiense*. The animals were killed at various times after infection, serum prepared and stored at -70C until used. Specific isotype responses were assayed by indirect fluorescence (FA). IgM antibody was detected in CBA/CaJ on day 6, in infected nude mice on day 8, and in CBA/N mice on day 14. A cross (CBA/N x Nu/Nu)F_1 nude mice infected with *T. rhodesiense* revealed specific IgM antibody at day 14, and none was detected prior to that time. IgG3 antibody was detected at the same time as was IgM, yet this response became undetectable after day 20, and appeared at brief intervals thereafter. These results suggest that the IgG3 is T-independent, and has a unique specificity for a determinant(s) on the surface of the parasite. The data further suggest that IgG3 actually is cyclic in nature, perhaps corresponding the antigenic variant in the trypanosome population.

Further IgM antibody reacted specifically with all stages of the parasite, whereas IgG2 had specificities associated with short stumpy forms. IgG3 reacted with all stages. Both IgG isotypes were detected during the first 3 weeks in enthymic mice, yet IgG3 disappeared, whereas IgG2 antibody persisted. Similar results were obtained with *T. b. brucei* indicating that the isotype response is not a parasite phenomenon, and is related directly to the host response *per se*.

Flow Cytometry

Other studies have shown an apparent non-specific adsorption of Ig and other serum proteins to the surface of African trypanosomes. Examination of trypanosomes (*T. rhodesiense*) by the FACS revealed no Ig, albumin, or C3 on the surface of the parasites. This was established by the use of class specific reagents, either the IgG or $F(ab')_2$ fractions. The former will bend to the parasites, the latter will not. Thus, non-specific reactions, reported elsewhere with IgG fractions of antisera, *reflect* the binding of these reagents *per se* to the trypanosomes, and as such yield false positives. Our results suggest that specific binding of antibody to African trypanosomes depends on the qualtiy of the detecting reagents, and will enable a more complete study of the specific isotype response of this infection.

Immune Complex Study

The presence of immune complexs in the blood of infected CBA and A/J mice was assessed by the 125I-Clq binding assay. The Clq binding assay was elevated 10 days after infection. After 28 days of infection, the Clq binding continued to rise in CBA/Ca T6 and A/J mice, while it stabilized in CBA/a mice. The continued use of Clq binding was associated with early onset of mortality in CBA/Ca T6 and A/J mice. No increase was detected in CBA/N mice, and no mortality was observed during the same period.

Publications:

Finerty, J. F., Rosenberg, Y., Kendrick, L. P. and McKelvin, R: B-cell activation in congenitally athymic and enthymic-mice infected with *Trypanosoma rhodesiense*. In, *Proceedings of the Third International Workshop on Nude Mice*, editor N. Reed, in press, 1981.

Gasbarre, L. C., Finerty, J. F. and Louis, J. A. 1981. Non-specific immune responses in CBA/N mice infected with *Trypanosoma brucei*. *Parasitol. Immunol.*, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00140-16 LMI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunology of Malaria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	M. Meltzer	Research Microbiologist	LIB/NCI
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Non-lethal malaria was injected into macrophage deficient and normal mice. Resistance was noted with deficient mice, whereas mortality was observed in normal mice. Results indicated that resistance was species specific and was dependent upon host response.

PHS-6040
(Rev. 2-81)

Project Description:

Antibody levels have little relevancy as indicators of host protection against malaria. Yet, thymus, T-cells and phagacytic cells are necessary for host protection in murine malaria. Whereas cell-mediated immunity has been studied in rodent malaria, the role of phagacytic cells is less understood. Our emphasis was to examine the effect of non-lethal malaria in strains of mice that were reported to have functionally defective macrophages e.g. inability to develop cytotoxicity in vitro.

Initial studies between C3H/HEN and C3H/HeJ mice infected with Plasmodium yoelii, indicated 75-80% mortality in normal C3H/HEN mice; C3H/HeJ showed only 5-10% mortality. Normal and deficient B10 mice, infected with same parasite cleared their infection. A pronounced difference was noted among the strains of mice in the time it took for parasite clearance. B10 mice cleared their parasitemia in 14-21 days, whereas C3H/HeJ in 28 days. NIH white mice clear P. yoelii 14-18 days. Preliminary studies with P. chabaudi in C3H mice indicated clearance and survival in both deficient and normal mice. In addition, infection of mice with P. yoelii or P. chabaudi does not lead to protection when challenged with the other species.

The ability of mice possessing macrophage deficiencies to clear both species of parasites in interesting since the deficiency is at a functional level i.e. inability to exhibit cytotoxic properties. The data suggest that cytotoxic deficiencies does not affect antigen presentation to T cells in the case of malarial infections. The lack of cross-protection between the 2 nonlethal parasites indicated that the response to either is specific to determinants not shared by either species. Further, the 2 strains of C3H mice responded differently to P. yoelii, yet apparently shared the same response to P. chabaudi indicating vast differences between these parasites. All strains of mice were susceptible to P. yoelii lethal strain. These results suggest that in assaying the genetics of the immune response to malaria, more than 1 species strain of parasite must be utilized.

Publication:

Finerty, J. F., L. Kendrick, and R. McKelvin. Chemical enhancement of protective immunity. In, Inadvertant Modification of the Immune Response editor I. M. Asher. Proceedings of the Fourth FDA Symposium, 1980. Pp. 144-146.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00141-07 LMI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immune Responses to Malaria and Related Intracellular Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Monoclonal antibodies were used to characterize and isolate antigens of the rodent malaria parasite Plasmodium yoelii. Those antigens restricted to the merozoite stage, which might be involved in cell penetration, were examined in detail. Studies showed that at least two antigens were present in all Sporozoa tested which possessed rhoptries and micronemes, but were absent in all parasites that lack these structures. New studies were initiated to follow the kinetics of antimalarial antibody formation during the course of self-limiting malaria using a quantitative isotype specific solid-phase radioimmunoassay (RIA). The appearance of protective antimalarial antibodies was compared with the amount of antibody present in the serum and the number of malarial antigens which induced the response. Protective antibodies were found to be present primarily in the IgG_{2a} fraction of hyperimmune serum.

Project Description:

During malaria infection the host produces antibodies (Abs) to a variety of parasite antigens (Ags). Neither the location, number, and specificity of the malarial Ags nor the kinetics and subclass(es) of protective malarial Ab are known. During the last year we have continued to use monoclonal Abs against the rodent malaria parasite, Plasmodium yoelii, to isolate and characterize malarial Ags. Special emphasis has been made to characterize Ags possessed by merozoites (the parasite stage which invades erythrocytes) which might be involved in erythrocyte penetration. Studies following the kinetics of antimalarial Ab formation were initiated. A series of experiments were performed to determine - 1) the titer of IgM and the four subclasses of IgG in the serum of infected mice, 2) when protective Abs appeared in the serum, 3) the class and subclass of protective Ab and, 4) the number of Ags to which the host response during infection, and their correlation with protective immunity.

Use of Monoclonal Ab for Detecting and Characterizing Merozoite-Specific Ags

Twenty hybridoma cell lines have been studied in detail and found to identify at least 14 Ags. Three of these Ags are restricted to merozoites, that is, they are not found in earlier erythrocytic stages of the parasite. Indirect immunofluorescent studies show that 2 of the Ags are located in the apical (anterior) region of merozoites. These Ags are not only present in merozoites of P. yoelii, but also merozoites of all other rodent (P. berghei, P. vinckei, P. chabaudi), primate (P. knowlesi, P. falciparum) and avian (P. lophurae) malarias tested. We hypothesized that these Ags might be associated with the rhoptries (paired organelles) or micronemes which are located in the apical region of merozoites. Since other members of the class Sporozoa possess rhoptries and micronemes, we sought to determine if these monoclonal Abs would cross react with Coccidia. Both monoclonal Abs bound to the anterior portion of Toxoplasma gondii tachyzoites, Eimeria tenella and E. acervulina sporozoites and Sarcocystis bovis canus zoites. Additionally, all protozoa tested which lacked rhoptries and micronemes were negative. Electron microscopic (EM) studies (currently being undertaken with Drs. S. Langreth and H. Danforth) are required to identify the exact location of these Ags. Currently the function of these organelles is unknown but is thought to be involved in cell penetration. If EM studies demonstrate that the Ags identified by the monoclonal Abs are associated with apical structures, studies will be initiated to isolate and evaluate the biologic importance of these Ags.

Humoral Immune Responses of Malaria Infected BALB/c Mice

The immune responses of BALB/c mice infected with the nonlethal substrain of P. yoelii were examined during the course of infection. Initially an isotype specific solid-phase radioimmunoassay (RIA) was developed. This assay system was used to follow the kinetics of IgM and subclasses of IgG Ab formation. By day 6 after initiating infection, IgM and IgG_2 antiparasite Abs could be detected in serum whereas IgG_3 and IgG_1 Abs appeared later (days 9 and 11 resectively). Using a quantitiative isotype-specific RIA we found that the concentration of antimalarial Abs remained low (<50 μg/ml) in animals with patent parasitenias, but rapidly reached milligram levels (approximately 0.3 mg/ml IgM, 0.5 mg/mil IgG_1, 2.0 mg/ml IgG_2 and 0.5 mg/ml IgG_3) following the decline of paraistmia. On secondary infection a 1-2 fold increase in the amount of IgG_2 occurred, but only a slight increase in the other IgG subclasses was noted.

To evaluate the protective value of the IgG subclasses, hyperimmune serum (serum from mice infected many times) was fractionated by protein-A column chromatography. Hyperimmune serum and isolated IgG subclasses were passively transfered to mice challenged with 17XL P. yoelii. Saline control and mice receiving IgG_1, IgG_{2b}, and IgG_3 Abs died in 6 days, but mice receiving hyperimmune serum and IgG_{2a} Abs survivied for 20 days. Thus, the IgG_{2a} fraction was as protective as hyperimmune serum. One can hypothesize that IgG_{2a} Abs have a unique immunochemical property that makes them important in immune protection or that protective Ags induce primarily an IgG_{2a} response. To explore the latter hypothesis, the technique of immune precipitation was employed. In these studies, extracts of P. yoelii, metabolically labelled in vitro with ^{35}S-methionine or ^{3}3H-leucine, were incubated with purified Abs, precipitated with protein A sepharose, separated by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography. Numerous (over 20) Ags are recognized by all classes of immunoglobulins, but several Ags are restricted to either the IgG_1 or IgG_{2a} subclasses. Further resolution using 2-dimensional analysis (currently in progress) is required before Ags recognized exclusively by IgG_{2a} Abs can be characterized. However, these results suggest that protective Abs are associated with a single subclass of IgG. Hopefully, further studies will lead to the identification of protective Ags and/or the mechanism of Ab elimination of malarial parasites.

Publications:

Kim. K. J., D. W. Taylor, C. B. Evans, and R. Asofsky. 1980. Radioimmunoassay for detecting antibodies against murine malarial parasite antigens: monoclonal antibodies recognizing Plasmodium yoelii antigens. J. Immunol. 125:2565-2569.

Taylor, D. W., K. J. Kim, P. A. Munoz, C. B. Evans, and R. Asofsky. 1981 Monoclonal antibodies to stage-specific, species-specific, and cross-reactive antigens of the rodent malarial parasite, Plasmodium yoelii. Infect. and Immun. 32:563-570.

Taylor, D. W., and J. Rener. 1981. Production of antimalarial monoclonal antibodies. In, The Use of Nuclear Methodology and Techniques in the Study and Control of Parasitic Diseases of Humans. IAEA Publications, in press.

Taylor, D. W., P. A. Munoz, K. J. Kim, C. B. Evans and R. Asofsky. 1981. Plasmodium yoelii: comparison of indirect immunofluorescence and radioimmunoassay for detecting monoclonal antibodies to malaria. Exp. Parasit. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00142-08 LMI

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Development of Thymus-Derived (T) Suppressor and Amplifier Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Head, Microbiology and Immunology Section	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during current year.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00143-12 LMI

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Antibody Response to Type III Pneumococcal Polysaccharide (SSS-III)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	P. J. Baker	Head, Microbiology & Immunology Section	LMI/NIAID
OTHER:	B. Prescott*		
	G. Caldes	Chemist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	P. W. Stashak	Microbiologist	LMI/NIAID
	C. F. Hansen**		

COOPERATING UNITS (if any)

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**Small Animal Section, VRB, DRS, NIH, Bethesda, Maryland 20205

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	0.7	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

About 58 recombinant-inbred strains of mice have been developed from crosses between (FSxZB), (B6xCA), and (B6xC3) progenitor strains. A preliminary survey of their capacity to make an antibody response to α1,3-dextran and Type III pneumococcal polysaccharide (SSS-III) shows a wide spectrum of responsiveness. These strains should be useful in definitive studies on genes controlling responsiveness to these and perhaps other antigens.

Project Description:

Detailed studies conducted with Bailey recombinant-inbred (RI) strains of mice have shown that multiple genes acting in a complementary (additive) and independent manner influence that magnitude of the antibody response to Type III pneumococcal polysaccharide (SSS-III), α1-3, dextran and other bacterial antigens. None of these genes appear to be linked to the IgC_H or H-2 major histocompatibility locus; the high frequency of unique phenotypes observed in studies with other RI strains suggests that these genes are located on different chromosomes.

Over the past 2-3 years, at least 58 separate RI strains of mice, originating from (FSxZB), (B6xCA) and (B6xC3) background progenitor strains, have been prepared as part of long-term effort to isolate the genes involved in producing these effects and to obtain information on their mode of interaction; some of these strains are now in the F_{20} generation. The results of preliminary surveys revealed a wide spectrum of responsiveness to SSS-III and α1-3 dextran in these mice; low, intermediate and high responsiveness to both antigens were noted; a high response to one antigen was not associated with high responsiveness to the other conversely, indicating the segregation of "antigen-specific" immune response genes. A more detailed analysis of these immune response patterns is underway using these and other antigens; studies involving genetic control of regulatory T cell functions in these RI strains are also contemplated.

Publications:

Baker, P. J., Amsbaugh, D. F., Stashak, P. W., Caldes, G., and Prescott, B.: Immunological characteristics and central mechanisms involved in the antibody response to Type III pneumococcal polysaccharides. Revs. Infectious Diseases, 3:332-341, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 A1 00144-17 LMI

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of the Antibody Response to Pneumococcal and other Polysaccharide Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	P. J. Baker	Head, Microbiology & Immunology Section	LMI/NIAID
	J. Hiernaux	Visiting Fellow	LMI/NIAID
OTHER:	B. Prescott*		
	C. Bona**		
	C. DeLisi***		
	J. A. Rudbach****		

COOPERATING UNITS (if any) *Biomedical Research Institute, Rockville, Maryland 20852
**Mount Sinai Medical School, New York, New York
***Laboratory of Theoretical Biology, NCI, Bethesda, Maryland 20205
****University of Montana, Missoula, Montana

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Bacterial levan (BL) induces an immune response in mice in which a large fraction of antibodies express a cross-reactive idiotype shared with inulin-binding myeloma proteins. The percentage of anti-BL antibodies expressing the cross-reactive idiotype decreases with time after immunization, whereas the magnitude of the auto-anti-idiotypic response increases. This suggests that auto-anti-idiotypic antibody can suppress the complimentary anti-BL response. Athymic mice are not able to mount an auto-anti-idiotypic response; but, they produce an antibody response to BL that shows a cyclic (oscillatory) pattern. This indicates that auto-anti-idiotypic antibodies are not the main element in causing oscillations. However, they do modulate the expression of cross-reactive idiotypes in euthymic mice and there is no relationship between the suppressive property of auto-anti-idiotypic antibodies and the induction or maintenance of tolerance.

PHS-6040
(Rev. 2-81)

Project Description:

Since Jerne developed the concept of immune networks, the role of idiotypic interactions in regulating the immune response has been studied extensively for a variety of antigenic systems. Deliberate perturbations of the idiotypic equilibrium after inculation of a given idiotype or from the injection of specific anti-idiotypic antibody, can lead to the suppression or to the expansion of clones expressing the corresponding idiotype. Since the idiotypic network is assumed to be functional, regulatory auto-anti-idiotypic antibody or cells should be detected during the course of an immune response and their occurrence should be related to the generation of tolerance or immune memory. In fact, the detection of auto-anti-idiotypic antibodies has been reported in various experimental systems. Usually, their occurrence coincides with a decrease in the expression of the corresponding idiotype, which seems to indicate that auto-anti-idiotypic antibodies tend to suppress the immune response. Suppression by anti-idiotypic antibodies in particularly interesting because it suggests one of several mechanisms for the induction and/or the maintenance of tolerance, as well as a natural feedback mechanism that could explain the generation of oscillations in the number of antibody secreting cells (ASC) that occur in response to various T independent antigens. If the negative feedback exerted by auto-anti-idiotypic antibodies were the central factor responsible for the generation and the regulation of cyclic immune responses, one should not observe the latter in athymic nude (nu/nu) mice, which are undable to mount an auto-anti-idiotypic response.

The dynamics of the immune response to bacterial levan (BL) were investigated to provide information bearing on these possibilities. BL induces in BALB/c mice a T-independent immune response characterized by the production of antibody molecules of 2 different general types. One group of molecules is specific for inulin, a $\beta(2\rightarrow1)$ polyfructosan, as well as for BL, a $\beta(2\rightarrow6)$ polyfructosan with $\beta(2\rightarrow1)$ branch points; such antibodies express cross-reactive idiotypes (IdX-A, IdX-B, and IdX-G), which are shared with myeloma proteins that bind both inulin and levan. The second principal group of antibodies binds BL, but not inulin, and fails to express cross-reactive idiotypes (IdX) of anti-inulin antibodies. The plaque-forming cell (PFC) assay can be used to detect simultaneously the anti-BL response and the auto-anti-idiotypic response directed against the IdX/s expressed by anti-BL antibodies. Since the anti-BL PFC response is oscillatory, in order to investigate the possible role of auto-anti-idiotypic antibody in the generation and the regulation of this cyclic pattern, we have studies systematically the kinetics of the anti-BL and anti-E109Id, i.e., part of the auto-anti-idiotypic, PFC responses. The E109Id corresponds to IdX-A and IdX-G (22); it is shared by the levan-binding myeloma protein EPC 109 (E109) and a fraction of BALB/c anti-BL antibodies. Our results indicate that the relationship between the anti-BL and the auto-anti-E109Id responses is not simple. However, the fact that the percentage of anti-BL PFC expressing the E109Id decreases during the course of the response suggests a regulatory role for auto-anti-idiotypic antibody. To further characterize the BL antigenic system, we studies both the anti-BL and the anti-E109Id PFC responses in BALB/c nu/nu mice. The dynamics of the anti-BL response shows pronounced oscillations in nude mice despite the fact that an auto-anti-idiotypic response could not be detected.

High doses of BL induce an irreversible tolerant state. The paralysis or the deletion of specific B cells making antibodies specific for BL has been invoked to explain the establishment of this unresponsive state. Since auto-anti-idiotypic antibodies can suppress the production of anti-BL antibodies, it might be argued that part of the maintenance of the tolerant state depends on this suppressive mechanism. Consequently, we decided to examine whether mice tolerized with large doses of BL produce larger numbers of auto-anti-idiotypic (anti-E109Id) PFC than would be found during the course of an immune response to lower doses of BL and if the injection of an optimally immunogenic dose of BL modifies the magnitude of the auto-anti-idiotypic PFC response in tolerant animals. Neither was found to be the case.

Publications:

Hiernaux, J.: Network regulatory mechanisms of the immune response. In, Bona, C. and Cazenave, P. A. (Eds).: Regulation of Function of Lymphocytes by Antibodies, New York, Wiley p. 269-297, 1980.

Hiernaux, J.: Anti-idiotypic networks, Fed. Proc. 40: 1484-1488, 1981.

Hiernaux, J., C. Bona, and P. J. Baker: Neonatal treatment with low doses of anti-idiotypic antibody leads to the expression of a silent clone. J. Exp. Med. 153:1004-1008, 1981.

Bona, C., and J. Hiernaux: Immune response: idiotype anti-idiotype network. CRC Critical Review in Immunology 2:33-80, 1981.

Hiernaux, J., P. J. Baker, and C. Delisi: Modulation of the immune response to lipopolysaccharide. In, J. Hiernaux and C. Delisi (Eds). Regulatory Implications of Oscillatory Dynamics in the Immune Response, CRC Press, Inc. New York, 1981 in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00145-14 LMI

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mode of Action of Thymus-devived (T) Suppressor and Amplifier Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	P. J. Baker	Head, Microbiology & Immunology Section	LMI/NIAID
OTHER:	P. W. Stashak	Microbiologist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	G. Caldes	Chemist	LMI/NIAID
	B. Prescott*		

COOPERATING UNITS (if any)

*Biomedical Research Institute
Rockville, Maryland 20852

LAB/BRANCH
Laboratory of Microbiol Immunity

SECTION
Microbiology & Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Prior treatment (priming) with a sub-immunogenic dose of Type III pneumococcal polysaccharide (SSS-III) results in the development of an antigen-specific state of unresponsiveness termed low-dose paralysis. Such unresponsiveness can be transferred by spleen cells obtained from mice within 5-24 hrs after priming; the suppressive activity of primed cells is abolished by treatment with monoclonal antibody specific for the Thy 1.2 alloantigen in the presence of complement. These findings clearly show that low-dose paralysis is mediated by thymus-devived (T) suppressor cells.

PHS-6040
(Rev. 2-81)

Project Description:

Since we first reported that prior treatment (priming) with a sub-immunogenic does of Type III pneumococcal polysaccharide (SSS-III) results in profound immunological unresponsiveness (low-dose paralysis), several studies were conducted to define the mechanisms involved in producing this effect. Low-dose paralysis could be induced with all doses of SSS-III, whether or not priming elicited a detectable antibody response; it could not be overcome simply by the administration of a supra-optimal does of antigen. More important, CBA/N mice do not make a detectable serum antibody response to SSS-III; yet, low-dose paralysis can be induced easily in these mice. These findings negate the possibility that low-dose paralysis is due to antibody-mediated feedback inhibition. Because most (>90%) of the antibody made by mice given an optionally immunogenic dose of SSS-III is of the IgM class, one might argue that low-dose paralysis represents a "shift" from IgM to IgG antibody production; this was examined and not found to be the case.

Low-dose paralysis could be abolished by treatment with T cell depleting agents and could not be induced in athymic mice which otherwise give a normal antibody response to SSS-III; these observations suggested that the phenomenon is T cell dependent. The kinetics for Concanavalin -A- induced suppression, which is known to be caused by mitogen-activiated suppressor T cells, parallel those for low-dose paralysis thereby providing additional support for such a view. Although these findings also rule out the involvement of terminal or exhaustive differentiation and are consistent with the concept that suppressor T cells are responsible for eliciting the unresponsiveness observed, more direct evidence for this view was lacking. The results of current studies provides such evidence and clearly show that (a) low-dose paralysis can be transferred by spleen cells obtained from primed mice, and that (b) the suppressive effects produced by transferred cells can be abolished by treatment with monoclonal antibody specific for the Thy 1.2 alloantigen. Since low-dose paralysis is both induced and transferable within a short period of time (5-24 hrs) after exposure to SSS-III alone, there is no doubt that suppressor T cells play an important role in regulating the magnitude of the antibody response to this antigen which peaks, 5 days after immunization.

The results of detailed kinetic studies indicate that suppressor T cells are activated early (<18 hrs) during the course of a normal antibody response to SSS-III; this is consistent with data on the kinetics for the induction of low-dose paralysis and the fact that low-dose paralysis can be transferred with spleen cells collected within 5-24 hrs after priming. Suppressor T cell activity is highly antigen-specific and functions mainly to limit the extent to which clones of B cells proliferate (expand) in response to antigen. However, amplifier T cells are also involved in regulating the magnitude of the antibody response to SSS-III and other antigens. These cells, which differ from helper T cells in a number of respects, are activated later (2-3 days) after immunization with SSS-III and act in an opposing manner to drive antigen-stimulated B cells to proliferate further in response to antigen. Although it is not yet known whether both types of regulatory T cells are activated by antigen directly, the competitive nature of the effects produced by suppressor and amplifier T cells, as well as differences in the times at which their activities are expressed after exposure to antigen, could account in part for the difficulties we have experience in transferring low-dose paralysis with cells obtained at later intervals (2-3 days) after priming. This issue is now being investigated in conjunction with studies designed to examine the Lyt phenotype of both types of regulatory T cells, as well as their mode of interaction.

Publications:

Baker, P. J., Stashak, P. W., Amsbaugh, D. F., and Prescott, B.: Specificity of suppressor T cells activated during the immune response to Type III pneumococcal polysaccharide. Advances in Basic and Clinical Microbiology and Immunology, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00146-08 LMI

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunological Studies on Components Isolated from Bacteria, Parasites and Plants

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	P. J. Baker	Head, Microbiology & Immunology Section	LMI/NIAID
OTHER:	B. Prescott*		
	G. Caldes	Chemist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	P. W. Stashak	Microbiologist	LMI/NIAID

COOPERATING UNITS (if any)

*Biomedical Research Institute, Rockville, Maryland 20852

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology & Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.4	PROFESSIONAL: 0.4	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A water soluble polysaccharide from Carthamus tinctorius flowers was found to react with antisera specific to Types III and Types VIII Streptococcus pneumoniae. Introperitoneal inoculation of mice with this polysaccharide caused the generation of splenic antibody-forming cells, similar in specificity to those detected after immunization with Type III pneumococcal polysaccharide (SSS-III). Antiserum reactive to SSS-III was prepared by immunizing rabbits with C. tinctorius polysaccharide. Chemical analysis of the polysaccharide showed it to consist of xylose, fructose, galactose, glucose, arabinose, rhamnose and uronic acid. The analysis of the types of uronic acids present was facilitated by the development of a new chromatographic procedure, capable of differentiating between glucuronic and galacturonic acids.

PHS-6040
(Rev. 2-81)

Description of Project:

Many polysaccharides from natural sources have been found to react with anti-pneumococcus sera of various specificities; such cross reactivity has been used to study the structural basis for the development of specific immunity. These cross-reactive polysaccharides might be of great value in the preparation of effective vaccines against pneumococcal infections, as well as in basic studies on mechanisms involved in the induction of tolerance and the activation of regulatory T cell functions. Many plant polysaccharides have been prepared in our laboratory and have been characterized as to their chemical and immunological properties. Most recently, we observed that a polysaccharide isolated from the safflower (*Carthamns tinctorius*) is very similar to Type III pneumococcal polysaccharide (SSS-III) in its immunological properties. In fact, the "active" safflower polysaccharide may take the place of the latter in a variety of immunological procedures including simple precipitation and Ouchterlony tests with antiserum specific for SSS-III, production of antibody in mice reactive to SSS-III, and immunization of rabbits with the production of antiserum with a high precipitation titer to SSS-III. However, the "active" safflower polysaccharide was different in its dose-response characteristics in that up to 10 times more safflower polysaccharide was required to produce the same immunogenic effects as SSS-III.

Although SSS-III is composed solely of glucose - glucuronic acid repeating units, safflower polysaccharide was found to contain xylose, fructose, galactose, glucose, arabinose, rhamnose and uronic acids; evidently, glucose - glucuronic acid subunits are linked to a backbone structure composed of the remaining sugars present. A new chromatographic procedure was developed which permits on to separate and identify the kinds of uronic acids present.

Publications:

Caldes, G., Prescott, B., Thomas, C. A. III, and Baker, P. J.: Characterization of a polysaccharide from *Carthamus tinctorius* that Cross Reacts with Type III Pneumococcal Polysaccharide. J. Appl. Microbiol Immunol., in press.

Caldes, G., Prescott, B. and Baker, P. J.: The use of DEAE cellulose paper in the paper chromatographic separation of uronic acids. Jour. Chromatography, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00153-05 LMI

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

In Vitro Response of Human Peripheral Lymphocytes to Infectious Organisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Asofsky	Laboratory Chief	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

No progress was made in the past year.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00158-05 LMI

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Immune Response to Entamoeba Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.F. Finerty	Research Microbiologist	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Immunity

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NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during currrent year.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00186-08 LMI

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Autoimmunity in Inbred Strains of Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.M. Chused	Senior Investigator	LMI/NIAID
OTHER:	H. C. Morse III	Senior Investigator	LMI/NIAID
	W. F. Davidson	Expert/Consultant	LMI/NIAID
	V. Manohar	Senior Investigator	LMI/NIAID
	H. A. Wilson	Guest Worker	LMI/NIAID
	E. Brown	Biologist	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Virology and Cellular Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.3	OTHER: 0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The immunologic mechanism and genetic control of autoimmune disease is being investigated in autoimmune mice utilizing flow microfluorometry and functional analysis of lymphocyte subpopulations.

PHS-6040
(Rev. 2-81)

Project Description

The long term goal of this laboratory is to advance our understanding of the molecular basis of autoimmune disease so that (a) safer, more specific, therapy may be designed, and (b) our knowledge of immunoregulation will be enhanced. Although several inbred strains of mice spontaneously develop autoimmune disease, our efforts are concentrated on the New Zealand Black (NZB) mouse. The NZB mouse was the first autoimmune strain described and it remains the one most similar to human disease. In recent years, we and other laboratories have shown that NZB mice have a primary B cell abnormality. Their spleen cells spontaneously secrete 40 times as much pentameric IgM as spleen cells from normal mice; have ten times as many IgM containing plasma cells; and have ten times as many PFC to several common haptenes. These abnormalities are detectable at birth and are present in *nu/nu* and T deprived NZB mice. These observations indicate that NZB mice develop polyclonal activation of B cells.

NZB mice also express a primary T cell abnormality, namely a primary cytotoxic T cell response *in vitro* to H-2 compatible mice. Suppression of B cell activity with anti-IgM antibodies indicates that the CTL response is independent of B cell activation. Thus, the critical question is the relationship of the T and B abnormalities to each other and to the autoimmune disease process.

Genetic analysis of (NZB x B10.D2) x B10.D2 backcross mice for T and B cells abnormalities confirm the independence of these traits. The B and T abnormalities segregate independently. Furthermore, the CTL defect depends on the presence of two NZB genes, at least one of which is semi-dominant. One of these genes is located on chromosome 1 near Ly 9 (also known as Lgp 100). In this cross the B cell abnormality appears to depend on one gene which is distinct from the two T cell genes.

The work in progress is directed toward identifying the abnormal T cell, i.e. the Ly 2^+ CTL precursor or the Ly 2^- helper. In addition, we are carefully examining the cell populations present in NZB lymphoid tissues with age. To this end we have prepared rat-mouse hybridomas with spleen cells from Lewis rats immunized with NZB spleen cells. The first two such reagents recognize H-2 antigens, one Ia^d and the other several K and/or D specificities. We are utilizing a panel of hybridomas to B, T, macrophage and null cell differentiation antigens as well as several reagents which differentially stain various cell types. Finally, we are examining NZB spleen cells with charge sensitive dyes and ionophores in an attempt to get closer to the underlying cellular abnormality.

Publications

Davidson, W. F., Chused, T. M. and Morse, H. C. III. Genetic and functional analysis of the primary *in vitro* CTL response of NZB lymphocytes to *H-2* compatible cells. Immunogenetics 12:445-463, 1981.

Davidson, W. F., Chused, T. M. and Morse, H. C. III. 1981. Genetic control of B- and T- lymphocyte abnormalities of NZB mice. Immunogenetics, 13:421-434.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00187-08 LMI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies in Sjogren's Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. M. Chused	Senior Investigator	LMI/NIAID
OTHER:	E. M. Brown	Biologist	LMI/NIAID
	H. Moutsopoulos	Visiting Investigator	LMI/NIDR
	T. Lawley	Senior Investigator	D/NCI
	D. Mann	Senior Investigator	I/NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Virology and Cellular Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during current year.

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(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00131-14 LMI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Hypersensitivity in Inbred Histocompatible Guinea Pigs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Stone	Head, Experimental Autoimmunity Section	LMI/NIAID
OTHER:	R. B. Nussenblatt	Senior Investigator	CB/NEI
	C. S. Raine*		
	M. Gottlieb**		

COOPERATING UNITS (if any)
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**Department of Medicine, Division of Clinical Immunology, UCLA, Los Angeles

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Experimental Autoimmunity Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been studying allergic encephalomyelitis (EAE) in juvenile guinea pigs which can develop cataracts in the acute phase and will undergo a chronic stage which resembles multiple sclerosis in man. The cataracts, which develop from the lenticular cortex in acutely paralyzed juveniles, are being analyzed biochemically. In face of a reduction of soluble proteins in the lens, the proteins fround in the insoluble fraction are being examined. Cataract formation is not seen in EAE-resistant strain 2 guinea pigs nor in adults of any strain. The acute phase of EAE could be suppressed by cyclophosphamide or by injection of myelin basic protein during the incubation period. The juveniles treated in this way did not show cataracts, and remained cataract-free when they went on to a chronic EAE (which was not suppressed under the experimental conditions). With Dr. Gottlieb at UCLA we are attempting therapy of chronic EAE using massive serial doses of irradiation (total lymphoid radiation) and with Drs. Traugott and Raine at Albert Einstein analyzing the T-cells migrating to the CNS by use of monoclonal antibodies.

PHS-6040
(Rev. 2-81)

Suppression of the Acute Stage of EAE in Juveniles

The early and acute phase of EAE in young guinea pigs can be suppressed so as to provide a solely chronic disease more eeminiscent of multiple sclerosis. Two approaches were successful in this regard: 1) general immunosuppression by cyclophosphamide, and 2) specific suppression by prior injection of myelin basic protein without complete Freund's adjuvant. A third method simply using strain 2 EAE-resistant guinea pigs has been elusive with strain 2's sometimes showing resistance to both acute and chronic syndromes and other times about half of them undergoing typical chronic EAE with long incubation period. Large numbers of such chronicly affected animals are necessary for the sub-projects which are described below.

T-Cell Migration to Central Nervous System in EAE

Previous studies on lymphocytes isolated from the central nervous system (CNS) tissues of guinea pigs with EAE have shown that T cells migrate from the circulation to the meninges as clinical signs become manifest. In the present project, the lymphocyte dynamics within the CNS could be investigated since immunologic markers for T cells in sections of frozen CNS tissue were made available from Dr. Shevach's (Laboratory of Immunology) preparations. T and B cells were demonstrated in sections of brain and spinal cord from guinea pigs with acute and chronic EAE either non-suppressed or suppressed by the prior injection of myelin basic protein. A monoclonal anti-guinea pig T cell reagent in combination with fluoresceinated anti-mouse Ig was used to characterize T cells, and B cells were identified using rhodamine-conjugated anti-guinea pig IgG. While B cells usually remained localized in perivascular cuffs and meninges, T cells showed predelection for white matter. The density of infiltrated cells increased with disease severity, while in remission only scattered T cells were found in white matter and B cells were almost absent.

Total Lymphoid Irradiation of Guinea Pigs in Prevention and Therapy of Chronic EAE

With Dr. Michael Gottlieb's group at UCLA we are applying that group's approach to immunosuppression by total lymphoid irradiation of juvenile strains 13 quinea pigs prepared for chronic EAE. The Division of Clinical Immunology is interested in an application to patients with MS. Preliminary results indicate that when animals receive 200 rads/day to a total of 4,000 rads they are protected from chronic EAE. Low doses of azothiaprine will also be tried in conjunction with irradiation.

Characterization of Proteins in Cataractous Lenses of EAE-Affected Strain 13 Guinea Pigs

In strain 13 guinea pigs, the cataracts seen in acute EAE are being analyzed for changes in the lens proteins. These studies in collaboration with Dr. Nussenblatt and Dr. Zigler at NEI have had a promising beginning.

Components of the altered lens which warrant characterization might be found in opacification and remission. The advantages of an inbred animals with large size lenses for this type of investigation are self-evident. The cataract proteins of EAE-induced type will also be compared to those from strain 13 guinea pigs which have been bred from a colony we have set up starting with a female with "spontaneous" nuclear cataracts crossed with a normal male. A resultant cataractous male offspring has served as sire to our line. Preliminary results inducate a autosomal dominant trait is responsible for this congenital cataract which is different in many aspects from the experimentally induced opacities in EAE of juvenile guinea pigs.

Publications:

Stone, S. H., Nussenblatt, R. B., Cross, F. L., and Raine, C. S.: Cataracts and allergic encephalomyelitis: acute opacification of the lens in paralyzed juvenile guinea pigs. Apthalmic. Res. 13:129-138, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00203-02 LMI

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Applications of Flow Microfluorometry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. M. Chused	Senior Inivestigator	LMI/NIAID
OTHER:	W. M. Leiserson	Mathematician	LMI/NIAID
	V. Manohar	Visiting Fellow	LMI/NIAID
	H. A. Wilson	Guest Worker	LMI/NIAID
	E. Brown	Biologist	LMI/NIAID
	B. E. Seligmann	Research Associate	LCI/NIAID
	J. I. Gallin	Senior Investigator	LCI/NIAID
	G. Weil	Research Associate	LPD/NIAID
	C. A. Kozak	Staff Fellow	LVD/NIAID
	I. Levy	Computer Specialist	DCRT/NIH
	M. Wilkinson	Visiting Fellow	LIG/NIAID
	M. Berger	Research Associate	LCI/NIAID
	J. Smollen	Visiting Fellow	ARB/NIAMDD
	A. F. Steinberg	Senior Investigator	ARB/NIAMDD
	R. Hall	Senior Investigator	DB/NCI
	T. Lawley	Senior Investigator	DB/NCI
	M. Frank	Clinical Director	LCI/NIAID
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LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Virology and Cellular Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.6	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Flow microfluorometry is being applied to the study of charge sensitive dyes, the purification of human eosinophils, the detection of spontaneous antibody to murine leukemia viruses, somatic cell genetics the generation and analysis of hybridomas and analysis of lymphocyte subsets in human disease.

PHS-6040
(Rev. 2-81)

Project Description:

Flow cytometry is a powerful technique in cellular immunology. The recent development of a dual laser fluorescence activated cell sorter (FACS) permits the simultaneous measurement of light scatter (closely related to cell size for viable cells) and fluorescence in two independent channels at flow rates of up to 5000 cells per second. Single parameter analysis, typically using fluorescein-conjugated reagents, has become a fairly standard immunologic technique. The goal of the NIAID FACS laboratory is to provide as extensive collaborative opportunities as possible to investigators in the Institute as well as to explore new applications of this advanced technology. To this end a number of enhancements have been added to the computer software used with the FACS. Completely new programs have been written in a much more flexible and powerful computer operating system to collect and analyze FACS data. These store a complete description of the material being tested, staining agent(s), and operating parameters and make it available during data retrival and analysis. Each investigator's data is automatically archived for rapid retrival. Logarithmic amplifiers have been incorporated into the system as well as the software to handle this type of data. The data analysis program provides extensive capabilities of integrating, comparing, subtracting, manipulating and plotting data files. This program can be set up to procede automatically for large numbers of files. Advanced techniques such as emission anisotropy and list mode analysis have been implemented.

To date, thirty investigators have used the system. Some of the projects underway include:

1. The use of charge sensitive cyanine dyes to measure the membrane potential of lymphocytes and their response to various stimuli. We want to answer the question of whether membrane depolarization is an integral part of the process of lymphocyte activation. Other vital dyes that stain DNA and RNA are being investigated to develop a battery of reagents that will describe the functional state of a lymphocyte as well as monclonal antibodies describe their differentiative state. We the hope to utilize the multiparameter capability of the system to relate activation state to differentiation state. Lymphocytes from NZB mice, which are spontaneously activated are being used in these studies.

2. The purification of human eosinophils to 98+% purity on the basis of their spontaneous autofluorescence. Since the cells are viable and unstained they function normally in several assays. We have been able to determine that the fluorescent material in eosinophil granules is flavin adenine dinucleotide.

3. The isolation and cloning from mouse-hamster somatic cell hybrids of lines expressing different and distinct amounts of muring leukemia virus gp70 in an effort to map the responsible gene(s).

4. The development of a panel of rat anti-NZB hybridomas,

5. The analysis of a panel of mouse anti-rabbit T-cell tumor gylcoprotein hybridomas.

6. The measurement of the ratio of helper to suppressor T cells in patients with systemic lupus erythematosus and normal controls. This study showed that SLE patients have a wider range of $T_h:T_s$ than normals and that there are significant correlations between this ratio and certain clinical features. Patients with a low $T_h:T_s$ have more renal disease and thrombocytopenia and onset of disease before age 20 while patients with a high ratio had multisystem disease including lymphadenopathy but only rarely SLE renal disease.

7. Measurement of the uptake of biotinylated C'3 by senstized erythrocytes to show correlation between radioactivity and fluorescence and to determine the distribution of cellular uptake.

8. Determination of lymphocyte subsets in mice infected neonatally with Rauscher or Moloney leukemia virus. We are attempting to determine the susceptible cell type.

Publications:

1. Weil, G. J. and Chused, T. M. Eosinophil autofluorescence and its use in isolation and analysis of human eosinophils using flow microfluorometry. Blood 57:1099-1981.

2. Seligmann, B. E., Chused, T. M. and Gallin, J. I. Human neutrophil heterogeneity identified using flow microfluorometry to monitor membrane potential. J. Clin. Invest., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00204-01 LMI

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immune Responses to Non-H-2 Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wendy F. Davidson	Expert/Consultant	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Virology and Cellular Immunology

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project to be terminated.

PHS-6040
(Rev. 2-81)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00205-01 LMI

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Phenotypic and Functional Abnormalities of T and B Lymphocytes of Autoimmune Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. F. Davidson	Expert/Consultant	LMI/NIAID
	H. C. Morse III	Senior Investigator	LMI/NIAID
OTHER:	T. M. Chused	Senior Investigator	LMI/NIAID
	C. Kozak	Staff Fellow	LVD/NIAID
	B. J. Mathieson	Senior Staff Fellow	LMI/NIAID
	J. Roths*		
	E. Murphy*		
	R. Coffman**		

COOPERATING UNITS (if any)
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**Stanford University, Palo Alto, CA.

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Virology and Cellular Immunology

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Research has involved the study of the immunologic and genetic mechanisms involved in the development and expression of autoimmune disease. Two autoimmune strains of mice NZB and PN, have been used to study (a) phenotypic and functional abnormalities of T and B lymphocytes prior to and after the onset of disease symptoms and, (b) the genetic control of these abnormalities. NZB and PN B lymphocytes undergo spontaneous polyclonal activation characterized by a decrease in the ratio of cell surface IgD/IgM a marked increase in plasma cell frequency, an elevated rate of IgM secretion/plasma cell, and the production of autoantibodies specific for thymocytes and erythrocytes. In NZB mice, genetic studies showed that alterations in cell surface IgD expression and production of anti-erythrocyte antibodies are recessive traits, whereas plasma cell frequency, control of IgM secretion, and production of anti-thymocyte antibodies, are governed by one or more dominant or semi-dominant genes. NZB and PN T cells make an unexpected primary cytotoxic T cell response to H-2-compatible cells in vitro. In NZB mice this response is controlled by two genes one of which is linked to Ly9 on chromosome 1. The genes controlling T cell dysfunction are different from those controlling polyclonal B cell activation.

Project Description:

Systemic lupus erythematosis (SLE) in humans is a complex autoimmune disease characterized by the production of antibodies to nucleic acids and immune complex glomerulonephritis. The cause of SLE is unknown but a number of murine models for lupus now exist which provide the opportunity for detailed study of the pathogenesis of this disease. These models are particularly valuable for providing insights into the immunologic events which occur prior to the onset of clinical disease. Our research efforts have concentrated on three murine models of autoimmune disease; NZB mice, PN mice, and a number of strains of mice carrying the gene lpr which causes severe lymphoproliferative disease. These mice were evaluated at various ages for phenotypic and functional abnormalities of T and B lymphocytes.

NZB Mice. NZB mice develop spontaneous autoimmune disease characterized by the production of multiple autoantibodies. Although these antibodies are not present in high levels until the mice are between 5 and 12 months of age, our studies strongly suggest that their appearance reflects major derangements of B- and T- lymphocyte function which can be detected during the first month of life. Thus, NZB B lymphocytes undergo spontaneous polyclonal activation characterized by a decrease in the ratio of cell surface IgD/IgM, a marked increase in plasma cell frequency, and an elevated rate of IgM secretion/plasma cell. As all of these defects can be detected in the absence of T lymphocytes, they are felt to represent intrinsic abnormalities of NZB B cells.

NZB mice also exhibit a number of T-lymphocyte dysfunctions. Included among these is an unexpected primary cytotoxic T lymphocyte (CTL) response to H-2-compatible cells in vitro. We have shown that this unusual response can be detected by two weeks of age, does not require B lymphocytes or their products for its expression, and is directed at multiple antigens.

A question of major importance arising from these studies is whether there is a common genetic basis for some or all of the observed lymphocyte defects. This question was approached by studying progeny of crosses between NZB and B10.D2 mice before 3 months of age for (a) altered expression of cell surface IgD, (2) splenic plasma cell frequency, (3) rates of IgM synthesis/plasma cell, and (4) generation of a primary in vitro CTL response to BALB/c cells. At 10-11 months of age, the same animals were tested for circulating antibodies to red cells and thymocyte-binding antibodies (TBA). The results showed that in this cross, alterations of cell surface IgD expression and production of red-cell-specific antibodies are recessive traits, whereas plasma cell frequency, control of IgM secretion, production of thymocyte-binding antibodies, and the generation of primary anti-BALB/c CTL responses, are governed by one or more dominant or semi-dominant genes. Specifically, plasma cell frequency appeared to be controlled by a single semi-dominant gene, IgM synthesis rate by one or two dominant or semi-dominant genes, TBA by three or more independently segregating dominant genes and the CTL abnormality by two genes at least one of which was semi-dominant. The data also suggested that there may be a single locus common to the genes regulating B cell defects.

Backcross mice were also analyzed for segregation of alleles at Ly9, Qa-1, Pep-3 and Gpd-1. Comparisons of CTL activity with these markers in backcross mice showed a significant correlation between the ability to generate a strong anti-BALB/c CTL response and alleles at Ly9. Thus, one of the two genes governing this response maps to chromosome 1 in linkage with Ly9. Neither of the NZB CTL genes was associated with the NZB gene(s) governing polyclonal activation of B lymphocytes.

PN Mice. The newest and least studied model for SLE is the PN mouse. PN mice develop autoimmune disease characterized by the development of arteritis, glomerulonephritis, antibodies to DNA and lymphoproliferation. Mice six weeks to one year of age were studied for functional and phenotypic abnormalities of T and B lymphocytes. Cytotoxic T cell response to allogeneic cells were normal in 3-5 month old mice but were decreased in older mice. PN mice, like NZB mice, also generated a primary cytotoxic T lymphocyte response to H-2 compatible cells *in vitro*. However, in PN mice, this response was not detected after four months of age. Primary, *in vitro* plaque-forming cell responses to SRBC were normal in three month old mice but decreased with age thereafter. Spleen cells of young mice had increased numbers of plasma cells and the numbers increased with age. IgM was the predominant class of antibody secreted by plasma cells in young mice and IgG the predominant class in mice older than six months. With aging, the expression of both surface IgD and IgM on splenic B cells decreased progressively. The autoimmune characteristics of PN mice thus appear to resemble those of NZB and (NZB x NZW)F_1 mice in a number of respects and differ significantly in others.

lpr/lpr Strains. The mutant gene lpr (lymphoproliferation) is an autosomal recessive which results in the development of massive, general lymphadenopathy. Mice become hypergammaglobulinaemic, produce antibodies to DNA and thymocytes, and develop immune complex glomerulonephritis. The effects of this gene on the phenotype of T and B lymphocytes from the spleen and lymph nodes have been studied in the following congenic strains; AKR/J, SJL/J, NZB/J, C57Bl/J, and C3H/HeJ.

Each of these strains showed a marked decrease in the proportion of B lymphocytes in the spleen and lymph nodes detected by B cell-specific reagents reacting with surface Ig, thymus bone marrow antigen (ThB) and Lym20 and Lyb2 antigens. The phenotype of T lymphocytes was also abnormal. The proportion of $Lyt2^+$ spleen and lymph node cells was significantly decreased in SJL and NZB strains. By contrast the proportion of $Thy1.2^+$ cells in the spleen and lymph nodes was increased in all strains except B6.

Spleen and lymph nodes cells were also stained with a monoclonal reagent which reacts with all peripheral B lymphocytes and approximately 25% of bone marrow cells. The proportion of cells expressing this antigen was increased markedly in lpr/lpr lymph node and to a lesser extent spleen. The predominant abnormal cell population in lpr/lpr lymphoid organs, therefore, bear markers for both T and B lymphocytes. These cells may represent a subpopulation present in normal mice which is abnormally expanded in lpr/lpr mice or, alternatively, may represent a unique type resulting from abnormalitites in the differentiation process of lymphoid cells. Spleen cells from lpr/lpr mice gave significantly weaker CTL responses to allo-antigens than lpr/+ or +/+ mice suggesting that at least one functional subpopulation of T cells is affected by the lpr gene.

Publications:

Davidson, W. F., Chused, T. M. and Morse, H. C. III: Genetic and functional analyses of the primary in vitro CTL response of NZB lymphocytes to H-2-compatible cells. Immunogenetics 12:445-463, 1981.

Davidson, W. F., Chused, T. M. and Morse, H. C. III: Genetic control of B- and T- lymphocyte abnormalities of NZB mice in crosses with B10.D2 mice. Immunogenetics 13:421-434, 1981.

LABORATORY OF PARASITIC DISEASES

1981 Annual Report

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Laboratory of Parasitic Diseases
National Institute of Allergy and Infectious Diseases
SUMMARY - October 1, 1980 - September 30, 1981

INTRODUCTION

The major event during the past year regarding personnel for the Laboratory of Parasitic Diseases was the arrival of Dr. Alan Sher to head the Cell Biology and Immunology Section. Dr. Sher brought with him Guest Worker Stephanie James, Staff Fellow David Sacks and Visiting Fellow Andrew Simpson from England, so his group is off to a fine start. LPD now has a basic immunologist on its staff. Other evidence of moving LPD research into the area of molecular biology was the appointment of Senior Staff Fellow Thomas McCutchan with a background in recombinant DNA work and Staff Fellow John Dame. Because the NIH Associate Program has been taken out of the USPHS Commissioned Corps, our new physician coming to LPD for several years of research experience, Dr. Henry Francis, as well as future such personnel, will be referred to as Staff Fellows. New additions to Dr. Dvorak's Chagas' disease research program include Visiting Fellow Vera Bongertz from Brazil, WHO Fellow Dr. Miriam Postan and Guest Worker Juan Carlos Engel from Argentina. Research Associate Dr. Gary Weil left for what appears to be a fine faculty opportunity in St. Louis. Some new mother figure will have to be found for the Malaria Section to replace Visiting Associate Dr. Nava Epstein who returned to Israel. Our IPA program of collaborating investigators, which bloomed as quickly as a garden of many flowers, has just as quickly withered by the departures of Dr. Eskild Petersen to his faculty position at the University of Arizona, Dr. Edna Kaneshiro back to her job at Cincinnati, Dr. Phillip Smith's departure to LMI, and Dr. Robert Genta's imminent return to Hahneman Medical College in Philadelphia. The progressive decline in Intramural funding will not be able to support more than an occasional IPA investigator. Dr. Mark Crane, who has been a Visiting Associate, will be leaving for a position with industry and Staff Fellow Dr. Ron Rosenberg will be joining the Entomology Research Staff of WRAIR. We enjoyed very much the brief but productive stay of Guest Worker Dr. Niggi Weiss from Switzerland.

Dr. Mark Hoffstetter returned to Puerto Rico to complete his schistosomiasis immunological studies in collaboration with the CDC group. Dr. Ottesen is slated to go to India for filariasis work with his collaborators in Madras. Dr. Petersen made a quick trip to the Dominican Republic for follow-up studies on the diffuse cutaneous leishmaniasis patients there, and two of the patients were brought to the NIH Clinical Center for studies and treatment. Dr. Renato Gusmão, now a graduate student at Johns Hopkins and a Guest Worker with LPD, will initiate immunological studies on Chagas' disease patients in Brazil, based upon findings from the work that he and Dr. Neva recently completed in collaboration with Drs. Rezende and Rassi in Goiania, Brazil.

Laboratory of Parasitic Diseases
National Institute of Allergy and Infectious Diseases
SUMMARY - October 1, 1980 through September 30, 1981

RESEARCH ACCOMPLISHMENTS

MALARIA RESEARCH Surface Components of Asexual Malaria Parasites: An *in vitro* bio-assay has been discovered which should prove very useful in studying the important property deep vascular schizogony for *Plasmodium falciparum*. This is one of the events which leads to fatal outcome in infections with this type of malaria. Schizonts of *P. falciparum* were found to attach to a proportion of cells in cultures of human endothelium via the knob-like protrusions that occur on the infected cell membrane. The binding was specific for human cells and could be blocked by immune serum (Udeniya, Schmidt and Green of LI and Miller). Antigenic variation in *P. knowlesi* was found to be affected by absence of the spleen in the infected host. Schizont-infected cell agglutination (SICA) can be demonstrated with this species of monkey malaria, and can be utilized to demonstrate antigenic changes on the surface of infected cells if the course is prolonged. It was found, however, that if the parasite was passaged in splenectomized monkeys, SICA positivity, or the property of schizont agglutination by immune serum, was lost. It also appears that SICA negative lines of the parasite are less virulent for the rhesus monkey than SICA positive lines. It is postulated that the function of the variant antigen, and hence the SICA positive state, may be an evasion mechanism on the part of the parasite that requires interaction with the spleen to escape an immune response (Barnwell, Howard and Miller). In the process of trying to identify SICA positive antigens with various radiolabelling procedures, several other cell surface protein antigens that are very sensitive to trypsin have been identified (Howard and Barnwell). An important by-product of the SICA antigen work has been the use of cloning techniques for malaria parasites and improved methods for cryopreservation of parasites. (Howard, Barnwell and H.Coon of NCI). The degradation of hemoglobin by chloroquine-sensitive and chloroquine-resistant *P. berghei* is under investigation for clues to the metabolic basis of chloroquine-resistant malaria parasites. This system was chosen because the resistant rodent parasite fails to produce malaria pigment as it grows, (Hadley and Friedman of NIAMDD). Sporozoites of *P. knowlesi* and *P. cynomolgi* are being provided to other research groups for studies of monoclonal antibodies vs *P. knowlesi* and for infections of rhesus monkeys whose livers can be studied for latent exo-erythorcytic stages. (Gwadz with Nussenzweig in New York and Krotoski in New Orleans).

Sexual Stages of Malaria: The focus upon immunologic control over development of the sexual stages of the malaria parasite in the mosquito has disclosed some rather complicated relationships of the effects of avian serum upon *P. gallinaceum*, the chicken malaria parasite. Male and female gametocytes of this avian parasite are equally infectious for mosquitoes whether fed in the presence of heated or non-heated sera from a variety of avian hosts. However, the next stage in development after the fertilized macrogamete, the ookinete, is destroyed by fresh avian serum. There is evidence that this occurs via activation of the alternate complement pathway, but it does not occur normally in the course of parasite development because the labile complement components of serum are digested within 24 hours in the mosquito gut. Antibodies which block fertilization are not dependent upon complement for their effect. (Carter). Antigenic surface components of zygotes of *P. gallinaceum* have been identified by immune precipitation and surface labelling, and followed into the next developmental stage, the ookinete. Some of the components are shared by male and female gametes. But during transformation to ookinetes, all but one of six major components is lost and a new component of about 30,000 molecular weight appears. Events during this transformation period were also followed by examining a profile of immunoprecipitable components metabolically labelled with ^{35}S methionine. Thus, it has been possible to document antigenic and metabolic changes during the developmental stage of a malaria parasite. One of the components followed could be identified with a hybridoma antibody. (Kaushal and Carter). Hybridoma antibodies have been produced that react by immunofluorescence with gametocytes of *P. falciparum*. (Rener and Carter). Rhesus monkeys immunized over 4 years ago with *P. knowlesi* gametes in Freud's complete adjuvant continue to show transmission blocking immunity as evidenced by failure to infect mosquitoes after challenge infections. (Gwadz and Carter).

Physiology of Anopheline Mosquitoes: A requirement of erythrocytes, quite apart from nutritive value, for normal development of the malaria parasite within the mosquito has been found. Zygotes of *P. gallinaceum* when fed to mosquitoes free of erythrocytes failed to infect. If zygotes are injected directly into the hemocele of an unfed mosquito it will form oocysts. Therefore, it is postulated that the erythrocyte substance somehow facilitates invasion of the gut epithelium. (Rosenberg). Two anopheline species, *An. dirus* and *An. freeborni*, are under study for the stage of the cycle between oocysts and sporozoites in the salivary gland. Both species of mosquito support development to the oocyst stage with presence of mature sporozoites. Thereafter, the sporozoites invade salivary glands of *An. dirus*, but this does not occur with *An. freeborni*. (Collins and Gwadz)

MOLECULAR GENETICS OF PARASITES

Use of Recombinant DNA Techniques for Study of Parasites: A beginning has been made in LPD to use recently developed recombinant DNA technology to ask new questions and possibly exploit the new types of information about gene structure and expression in parasites. One of the systems being explored is the mouse malarial parasite, *P. berghei*. Clones and recombinants of *P. berghei* DNA have been produced and genomic DNA from different species of mouse malarial parasites can be distinguished. Some of the issues that we hope can be studied by this technology are the rearrangement of genes in a malarial parasite genome during development through various stages of life cycle and changes in gene arrangement during genetic crosses between related organisms. (McCutchan and Dame). The other system under study involves the helminth, *Schistosoma mansoni*. First, the DNA of this organism is being characterized. The *S. mansoni* genome is 2.7×10^8 nucleotide pairs in size and nearly half of the genome is composed of highly to moderately repeated sequences. Cloned genes from other organisms are being examined to see whether there are sequences in common with the parasite. (McCutchan and Simpson).

HELMINTHIC INFECTIONS

Pathogenesis and Treatment. Some of the early signs and symptoms of human filarial infections, such as recurrent episodes of lymphangitis (filarial fevers) and acute elephantiasis are probably manifestations of immune injury since they have been found to have high levels of circulating immune complexes. (Ottesen, Lunde and Lawley of NCI). The prenatal sensitization of babies born to microfilaremic mothers in India that was reported last year does involve presence of filaria-specific IgE antibody. (Weil, Hussain and Ottesen). An attractive hypothesis that would relate the extent of hepatic fibrosis of schistosomiasis to amount of fibroblast-stimulating factor recoverable from hepatic egg granulomas could not be supported in experimental animals (Cheever, Dean of NMRI and Dunn of WRAIR). The fate or clearance of one of the carbohydrate schistosome antigens, GASP, that originates from the gut of the worm and is constantly regurgitated into the circulation has been investigated in mice. Work from other groups has suggested that the GASP antigen is probably one of the main antigens deposited in the kidney in some patients with an immune-complex type of renal disease. Heavily infected mice had a slower clearance that those lightly infected, the liver was the main organ of clearance but some labelled GASP appeared in the urine as smaller size and non-antigenic material. (Nash). Longer term follow-up of the twelve Laotian refugees with S. mekongii infection and an additional few with S. japonicum has shown an excellent response and minimal side effects to Praziquantel treatment. This new anti-schistosomal drug is now available to our group at NIH. (Nash, Hofstetter, Ottesen and Cheever).

Immune response and regulation. Various techniques are being used to identify parasite antigens and specific classes of antibody reactive with these antigens. For example, three different assays for specific IgE (basophil histamine release, RAST and ELISA) were used to assess allergic sensitization in filariasis patients. (Ottesen, Hussain, Lunde). Parasite antigens which bind to human IgE are determined by crossed radio-immunoelectrophoresis (CRIE), a procedure in which first antigens and then hyperimmune sera are electrophoresed at right angles to precipitate in agar, then stained sequentially by the test serum, followed by radiolabelled anti-human IgE. Autoradiography by exposure of the dried gel to X-ray film will identify the antigen peaks reacting with IgE in the test serum. (Hussain). Excretory-secretory materials, or metabolic antigens, of parasites are usually less complex and may also exhibit more stage specificity than somatic worm antigens. Filarial and strongyloides metabolic antigens are being characterized, as well as IgE and IgG responses to them. (Hussain, Anwar). The significance of the "blocking antibodies" reported last year that inhibit allergic reactions has been extended; these are demonstrated by histamine release from basophils after exposure to parasite antigens. The blocking antibodies are IgG class molecules, occur in chronic human schistosomiasis as well as filariasis, may be present in high titer and appear to reflect an immune modulatory mechanism that correlates with lack of allergic manifestations in chronically infected patients. This inhibition of immediate hypersensitivity, which also extends to delayed T-lymphocyte reactions, was found to be reversed by treatment of schistosomiasis in patients chronically infected. (Ottesen, Hoffstetter). Immune mechanisms to experimental schistosome infection in mice act mainly at two points in the life cycle - 1) cercarial penetration in the skin and 2) later developing worms. A survey of infections in inbred mice showed variable degrees of resistance, but no linkage with major histocompatibility antigens. (Sher and James). Activated macrophages, evaluated by killing of tumor cells, probably play a role in immunity at the level of early developing worms. (James, Lazdins and Meltzer of NCI).

Factors involved in serodiagnosis. Various assays for IgE now permit diagnostic tests for parasite-specific IgE against broadly reactive as well as stage-specific parasite antigens. With sera from patients with filariasis, IgE antibody response was more specific than IgG responses. (Weiss, Hussain, Ottesen). In most patients only 1 or 2 percent of total IgE was parasite-specific, but in some instances it may be as much as 40 percent of total IgE. (Hussain, Ottesen). The ELISA test in patients with strongyloidiasis to larval (L3) antigen from *S. stercoralis* or *S. ratti* detected antibodies in 80 to 85 percent of parasitologically positive cases. The only important group of cross-reactions found thus far are in hyper-responsive, i.e., filariasis without microfilaremia patients. An IHA test with the *S. stercoralis* antigen is useful, but less specific than the ElISA. The fact that antibody levels decline within 6-12 months after treatment will make the test more useful clinically. (Neva, Gam and Burke of Kentucky).

INTRACELLULAR PROTOZOA

Analysis of clones of T. cruzi. Virtually neglected in research on *Trypanosoma cruzi* has been the issue as to genetic homogeneity of populations of the parasite. Biologic differences in a "strain" of parasites must certainly occur, on the basis of increased virulence for mice that often takes place on repeated passage. But whether these exist among the relatively small population of organisms that infects people, whether changes are induced in the host, or whether repeated exposure may introduce organisms with different properties are questions for which there are no answers as yet. By single-cell isolation 19 clones of *T. cruzi* have been isolated from two individuals with chronic Chagas' disease in Argentina. On the basis of growth rates, as epimastigotes in media, or as intracellular forms in culture, two populations of parasites with greatly different doubling times (24 vs 50 hours) have been isolated from each patient. (Dvorak and Argentinian collaborators). Possible immunologic differences among the clones is being studied (Bongertz and Dvorak), as well as pathologic changes produced in inbred mice. (Postan and Dvorak). Another approach to genetic differences in clones is that of flow cytometric analysis. An early model cell sorter has been rehabilitated and has been used to study subpopulations of *T. cruzi* clones for total DNA profiles at different stages of growth after staining with mithramycin. Differences of up to 30% in DNA content of cells from different clones have been found. (Dvorak).

Surface properties of T. cruzi. The ability of *T. cruzi* to infect cells in culture was found to vary with their stage in the cell cycle, i.e., G1, S, or G2. Ability to infect increased as cells went from G1 to S phase and decreased as they entered G2 phase. A similar pattern was found for toxoplasma parasites. One interpretation of these findings is that a cell surface component which varies in amount during the cell cycle acts as a receptor for the parasite. (Crane and Dvorak). As part of a general study on the role of the alternate complement pathway (ACP) as a natural defense against protozoan parasites, the effect of implanting different forms of the parasite in the host was investigated. Epimastigotes are known to be lysed by fresh serum, presumably complement, so a more gradual *in vivo* model was used, namely, subcutaneous implantation within a 0.45 μ millipore chamber which excludes cells. Surprisingly, after 24 hours organisms had converted from epimastigotes to trypomastigotes, which were now totally resistant to complement lysis. Transformation is strain dependent and requires log phase organisms. (Sher and Crane). Studies on anti-trypanosomal factor(s), ATF, produced by a bacterium, *Pseudomonas fluorescens*, have continued. The factor(s) consist of two fractions, the smaller of which is less toxic for mice and is probably a protein. The larger component is probably a polysaccharide. Main effects of ATF appear to be on membranes of the parasite. (Mercado).

New approach to immunity against parasites. A key immunologic phenomenon that thwarts host immunity to infection with African trypanosomes is the antigenic variation which the parasite develops in its outer membrane to evade immune attack. One approach is the use of monoclonal antibodies, but these will protect only against homologous antigenic type. The new approach is to immunize against the idiotypic determinants of several monoclonal reagents. Using anti-idiotypic antibodies as antigen, expression of the idiotype should theoretically be prevented. Preliminary results indicate that mice can be protected against *T. rhodesiense* infection by this maneuver. (Sacks and Sher).

Cell biology of leishmania. Detailed analysis of antigenic as well as biochemical components of leishmanial surface membranes is continuing with a variety of approaches. By immunization with Triton extracted *L. donovani* membranes and subsequent electrophoretic analysis, as well as by Iodagen labelling of surface membranes and later autoradiography of gel-separated samples, many antigens common to promastigotes and amastigotes were demonstrated. Further, sera from kala azar patients and from infected hamsters showed antibody to at least 8 of these components. (Dwyer). Using similar methods, the surface membranes of various strains of *Leishmania*, especially those associated with cutaneous disease have been examined and found to show a number of cross-reactive surface antigens. (Gardiner and Dwyer). Serum supplemented liquid media in which leishmanial promastigotes have been grown show antigenic materials (EF factor) that have been used in classification of leishmania. It seems fairly clear now that the EF factor is antigenic cell surface material that is shed into the medium during growth. One of the antigenic components may contain a lipid moiety containing inositol and present in other protozoa. (Kaneshiro, Gottlieb and Dwyer). Extractable lipids of *L. donovani* constitute 32 percent of the total cellular mass with the largest component being phospholipids. At least 3 lipolytic enzymes have also been identified in surface membranes. (Wassef and Fioretti). The acid phosphatase previously found in the pellicular membrane of L. donovani was also found to be detectable extracellularly, presumably released during growth of the parasite. (Gottlieb and Dwyer).

LUMINAL PROTOZOA

General growth and metabolism. We are a bit reluctant to keep reporting each year slight modifications in media for improving axenic growth of intestinal protozoa. Such efforts do not excite the molecular or cell biologist, yet these painstaking and usually empirical trial and error experiments have made and continue to make available these parasites for a wide variety of biochemical and immunological research. One small step forward has been a medium, No. 294, that substitutes for yeast extract and would thus facilitate study of nucleic acid metabolism of E. histolytica. Another medium, TYSGM-9, has been found highly efficient for initiating growth of a variety of lumen dwelling protozoa. (Diamond). The story of L-serine oxidation by E. histolytica in old TP-S-1 medium is not as simple as reported last year - under current conditions metabolism of this amino acid is much more feeble than it was years ago. (Weinbach and Keister). Progress with metabolic study of Giardia was limited mainly to two technical innovations, 1) the use of sepharose-bound dicourmarol in affinity chromatography to purify soluble oxidoreductases and 2) nuclear magnetic reasonance spectrophotometry to identify carbon-13 labelled glucose breakdown product in intact parasites. (Weinbach, Claggett and Costa of NIMH).

Giardia lamblia. Immuno-reactive antigens with human antibody are being defined by various techniques. (Smith, Nash). A number of putative immune and non-specific anti-parasitic reactions have been identified, but their significance in the human disease remains to be determined. One of the most promising mechanisms is direct cytotoxicity of monocyte-macrophage cells from peripheral blood for the parasite. Another mechanism involves antibody-dependent cell mediated cytotoxicity (ADCC) of granulocytes for the parasite, which is not complement dependent. (Smith, Keister). Fresh goat or rabbit anti-giardia sera killed parasites effectively, but human serum did not. Lactoferrin, an iron-binding protein present in intestinal secretions, can kill giardia,- but this probably does not operate in vivo because the parasites are protected by serum. (Gillin). Difluoromethylornithine (DFMO), an inhibitor of an enzyme involved in polyamine biosynthesis, has known activity against certain protozoa (Eimeria and African tryps.) but not against others (E.histolytica and T. vaginalis). However, DFMO is active against giardia, and this inhibition can be reversed by spermidine. (Gillin and P. McCann of Merrill Research).

Trichomonas vaginalis. The only accepted assay for virulence of Trichomonas vaginalis is the subcutaneous inoculation of mice for production of a local abscess. The possibility of using an in vitro assay, such as a toxic or cytopathic effect on cells in culture is being investigated. (Mattern). In a collaborative study with clinicians at Johns Hopkins, suspected metronidazole-resistant strains are evaluated for their drug susceptibility. Unfortunately, current laboratory methods are not entirely satisfactory for such in vitro-sensitivity tests. Clear cut resistance of T. vaginalis to metronidazole can only be demonstrated under aerobic conditions - under anerobic conditions the so-called resistant strains are susceptible to the drug. Extremes of pH will also influence growth of the organism. In spite of these problems the methods for evaluating susceptibility to metronidazole were useful in identifying resistant isolates from 4 patients who were successfully treated with I.V. metronidazole. (Mattern with Spence of Hopkins). The alternate complement pathway is activated by T. vaginalis to produce lysis of the organism, but the biological role of this defense mechanism is still not clear. (Gillin and Sher).

Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

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HONORS AND AWARDS

Dr. Richard Carter was again an invited teacher for the special parasitology course at Woods Hole, Massachusetts sponsored by the Rockefeller and McConnell Clark Foundations.

Dr. Allen Cheever serves on the Editorial Board of the American Society of Tropical Medicine and Hygiene.

Dr. Louis Diamond was an invited participant in a Workshop on Nutrition and Parasitic Diseases in Bellagio, Italy and to a WHO Scientific Group Meeting on Intestinal Parasitic Infections in Geneva.

Dr. Dennis Dwyer was recipient of the American Society of Parasitologist "Henry Baldwin Ward" Medal for 1980. He was also an invited speaker at the 1981 Gordon Conference on Parasitology, and was appointed to the Editorial Board of the Journal of Protozoology.

Dr. Teresa Mercado was an invited participant in a Conference sponsored by the MARC program, and as a Workshop leader at a Conference of Puerto Rican scientists sponsored by the American Association for Advancement of Science.

Dr. Louis Miller serves on Editorial Boards of Experimental Parasitology, Journal of Parasitology and Journal of Molecular Medicine. He is a member of the Steering Committee for Immunology of Malaria of the TDR of WHO.

Dr. Theodore Nash serves on the NIAID Clinical Research Committee..

Dr. Franklin Neva serves on the Editorial Board of the American Journal of Tropical Medicine and Hygiene, served on one of the STRC Review Committees of the TDR of WHO, and was an invited speaker at the 1981 Gordon Conference on Parasitology and was recipient of the Tenth Harry F. Dowling Lectureship Award from the University of Illinois College of Medicine.

Dr. Eric Ottesen is on the Editorial Board of Experimental Parasitology and serves on the Steering Committee of the Scientific Working Group on Filariasis of the TDR of WHO.

Dr. Eugene Weinbach serves on the Editorial Board of the Journal of Parasitology and was invited to write a review article for Trends in Biochemical Sciences.

PROJECT NUMBER (Do NOT use this space)	HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	Z01 AI 00092-15 LPD

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The pathogenesis of schistosome infections in mammalian hosts.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	T. E. Nash	Senior Investigator, LPD, NIAID
	E. A. Ottesen	Senior Investigator, LPD, NIAID
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	M. Barral	Kellogg Research Fellow, LPD, NIAID
	F. A. Sher	Senior Investigator, LPD, NIAID
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COOPERATING UNITS (if any) Southwest Foundation for Research & Education, San Antonio, TX (Dr. Kuntz); Dept. Gastroenterol., WRAIR (Dr. Dunn); Lab. Statist. & Math. Methodol., DCRT, NIH (Dr.Mosimann, Mrs. Minker, Dr. Malley); Div. Immunoparasitol., NMRI (Dr. Dean), American Foundation for Biological Research (Dr.Richards, Mr. Shade).

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host Parasite Relations

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Schistosome infections are studied quantitatively in man and experimental animals. Present studies emphasize fibrosis induced by schistosomiasis and resistance to reinfection with S. mansoni in laboratory mie.

PHS-6040
(Rev. 10-76)

Our studies examine the pathogenesis of schistosome infections in the mammalian host and immune reactions, as they relate to both pathologic reactions and to resistance to infection. Studies on pathogenesis currently emphasize the fibrotic response of the host to the eggs. Resistance to reinfection is examined in mice immunized through infection with S. mansoni or through exposure to irradiated S. mansoni cercariae.

Hepatic fibrosis. Previous results indicated great variation in hepatic fibrosis among different strains of mice infected with S. mansoni. The relative amounts of fibrosis were similar in acute and chronic infections, i.e., ICR mice had much more fibrosis than C3H mice, and C57Bl/6 mice showed less fibrosis than either of the other strains at 8, 12, and 20 weeks after infection. In a single experiment, two H-2b strains (C3H.B10 and C3H.SW) congenic with the C3H/HeSn strain showed a 4-fold difference in hepatic fibrosis.

As previously demonstrated by Wyler, extracts of hepatic granulomas stimulated the proliferation of fibroblasts in vitro. However, extract from the granulomas of animals developing marked fibrosis (outbred mice) were no more effective than extracts from animals developing minimal fibrosis (hamsters) during S. mansoni infection. These results suggest that the fibroblast simulating factor(s) in granulomas are not critical in regulating hepatic fibrosis in these models. Studies during the next year will examine the effects of liver extracts on rates of collagen synthesis and will examine the responses of fibroblasts from animals with high and low fibrotic responses to schistosome infection.

Resistance to reinfection by schistosomes in mice. Ten of the strains and substrains of S. mansoni developed by Richards were tested for the ability of bisexual infections to induce homologous and heterologous immunity. No tendence to stronger homologous resistance was present, but two important unexpected findings emerged. First, unisexual (male or female) infections with the PR-9 schistosome substrains consistently produce 50% resistance to challenge infections. Second, in a single experiment, the PRC-3 substrain induced poor (16-32%) resistance against all strains used while a second substrain from the same patient (PRT-3) induced excellent resistance (62-75%) against the same strains. In unrelated experiments in collaboration with Sher, mice immunized with irradiated S. mansoni cercariae were resistant to S. mansoni but not to S. japonicum.

Resistance induced by unisexual infections offer a much cleaner system for investigating immune mechanisms than do bisexual infections, as the latter are complicated by the effects of the host granulomatous response on migrating schistosomula. Differences in host responses to the poorly immunogenic PRC-3 substrain and the highly immunogenic PRT-3 substrain will allow much better discrimination concerning which host responses are important to resistance and which merely concommitant to infection. Finally, antigenic differences between these closely related substrains will be of great interest as potential immunogens. The failure of irradiated S. mansoni to immunize against S. japonicum offers similar advantages.

The humoral response of capuchin monkeys infected with S. mansoni displayed an exquisite species specificity to the homologous antigen. Much higher IgG antibody titers were obtained by an Enzyme linked immunosorbent assay (ELISA) when using adult worm or egg S. mansoni-derived crude saline extracted antigen than to similar S. japonicum preparations. On the other hand S. japonicum (Japanese strain)-infected capuchin monkeys recognized similarly antigens obtained from both parasites. Furthermore, such specificity was not found when both S. mansoni- or S. japonicum-infected monkeys were tested by lymphocyte proliferation tests in vitro. It is possible that all antigenic determinants recognized for antibody production, and revealed by the ELISA, on S. japonicum eggs and adult worms are also present in S. mansoni products, and the latter has additional determinants not shared with S. japonicum.

Humans infected with S. mansoni, however, do not show the same specificity in lymphocyte proliferation assays, although the IgG antibody titers are higher against the homologous antigens than to S. japonicum-derived ones. Humans infected with S. mekongi, a species closely related to S. japonicum, have higher lymphocyte transformation responses to S. japonicum antigens than to S. mansoni, although the IgG antibody titers were similar to both antigens, and similar results were observed in two patients infected with S. japonicum.

Publications:

1. Cheever, A.W., Duvall, R.H., and Minker, R.G.: Quantitative parasitologic findings in rabbits infected with Japanese and Philippine strains of Schistosoma japonicum. Am. J. Trop. Med. Hyg. 29: 1307-1315, 1980.

2. Cheever, A.W., Duvall, R.H., Minker, R.G., and Nash, T.E.: Hepatic fibrosis in rabbits infected with Japanese and Philippine strains of Schistosoma japonicum. Am. J. Trop. Med. Hyg. 29: 1327-1339, 1980.

3. Cheever, A.W., Duvall, R.H., and Minker, R.G.: Extrahepatic pathology in rabbits infected with Japanese and Philippine strains of Schistosoma japonicum and the relation of intestinal lesions to passage of eggs in the feces. Am. J. Trop. Med. Hyg. 29: 1316-1326, 1980.

4. Nash, T.E., Lunde, M.N., and Cheever, A.W.: Analysis and antigenic activity of a carbohydrate fraction derived from adult Schistosoma mansoni. J. Immunol. 126: 805-810, 1981.

5. Cheever, A.W., and Duvall, R.D.: Bladder calcification and obstructive uropathy in a gibbon infected with Schistosoma haematobium. Am. J. Trop. Med. Hyg. 30: 604-608, 1981.

6. Berman, J.D., Beaver, P.C., Cheever, A.W., and Quindlen, E.A.: A 5-cm cerebral cyst in a case of cysticerosis. Am. J. Trop. Med. Hyg. (in press).

7. Dean, D.A., Bukowski, M.A., and Cheever, A.W.: Relationship between acquired resistance, portal hypertension and lung granulomas in ten strains of mice. Am. J. Trop. Med. Hyg. (in press).

8. Wyler, D.J., Wahl, S.M., Cheever, A.W., and Wahl, L.M.: Fibroblast stimulation in schistosomiasis. I. Fibroblast stimulation in vitro by soluble products of egg granulomas. J. Inf. Dis. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00093-06 LPD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of the Immunologic Responses to Helminth Infections

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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R. Hussain	Collaborative Researcher (IPA), LPD, NIAID	
N. Anwar	Visiting Fellow, LPD, NIAID	
M. Barral	Visiting Fellow, LPD, NIAID	
N. Weiss	Visiting Scientist, LPD, NIAID	
M.N. Lunde	Research Zoologist, LPD, NIAID	
T.E. Nash	Senior Investigator, LPD, NIAID	
A.W. Cheever	Assistant Chief, LPD, NIAID	T.M. Chused
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COOPERATING UNITS (if any) Tuberculosis Chemotherapy Center, Madras, India (Dr. S.P. Tripathy); Medical College of Madras, India (Prof. K.V. Thiruvengadam; San Juan Laboratory, CDC (Mr. E. Ruiz-Tiben); Bureau of Veterinary Research, FDA, Beltsville, Md. (Dr.K.G. Powers); Johns Hopkins Medical Center (Dr. R.G. Hamilton)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host-Parasite Relations

INSTITUTE AND LOCATION
NIAID, Bethesda, MD. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
55/12	36/12	16/12

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The goals of this project are to characterize the host's immune response to helminth infections and to relate the findings to protective immunity and the pathogenesis of clinical disease. In filariasis susceptibility to infection appears partially genetically determined but unrelated to HLA. Experimental evidence indicates that IgE antibodies are important in protective immunity, and these antibodies are being quantified and immunologically characterized. IgE antibodies also appear to play a pathogenetic role in initiating inflammatory (allergic) reactions in tropical eosinophilia and may contribute along with immune complexes to the lymphatic inflammation leading to elephantiasis.

Chronic helminth infections are characterized by intense modulation of the host response to parasite antigens. Humoral and cellular suppressive mechanisms regulate lymphocyte responsiveness in human schistosomiasis and filariasis ans IgG blocking antibodies have been found to modulate immediate hypersensitivity responses. Following specific treatment the effects of these suppressive mechanisms is either diminished or lost.

Eosinophils are prominent in the host response to helminth infections. Purification of these autofluorescent cell by flow microfluorometry has permitted in vitro studies of their anti-parasite killing properties.

Project Description:

The character and evolution of the immune response in man and experimental animals has been studied during and following infection with parasitic helminths. Filariasis. The question of why some individuals in an endemic area become infected with filaria (W. bancrofti) while others appear to resist it and of why even among infected individuals the clinical manifestations of disease can be so varied have led to a search for the relevant immunologic parameters both in field studies of human filariasis and in work on laboratory models of the disease. Field studies have been carried out in the Cook Islands (Project #J0233I74) and in Madras, India (Project #76I231), this latter project continuing as an ongoing collaboration.

The issue of susceptibility to infection was studied after a significant familial clustering of filariasis was noted in the Cook Island population. Analysis showed this clustering to be most likely genetically determined - but not linked to HLAA or B markers. (Ottesen). Studies in an animal model have suggested susceptibility to infection to be associated with inadequate IgE antibody responses (Ottesen).

Efforts to define the determinants of the pathology seen across the clinical spectrum of filariasis have involved studies of lymphocyte responsivness, IgE and IgG antibody characterization and quantification and immune complex determination. At one end of the clinical spectrum is the tropical eosinophilia. (TE) syndrome of chronic lung disease with asthma and profound eoinophilia. Using three different assays for specific IgE antibody (basophil histamine release, RAST and ELISA) we have documented and quantified the high degree of allergic sensitization to filariae in patients with TE, compared to those with other forms of filariasis (Ottesen, Hussain, Anwar). The immunologic hyperresponsivness of these patients is not limited, however, to IgE antibody production but extends to IgG responses to a number of different parasite antigens as well (Anwar, Hussain, Nash, Ottesen). In other patients whose disease is manifested by elephantiasis and recurrent episodes of lymphangitis (filarial fevers) the finding of high levels of circulating immune complexes suggests a role for these complexes in the immune injury found in such patients (Ottesen, Lawley, Lunde).

The possibility that prenatal conditioning of the immune system, by altering the host's response to subsequent infection, may contribute to the heterogeneity of disease expression has been explored in studies of 60 pairs of maternal and cord blood collected in Madras. One fifth of the mothers (but no babies) were microfilaremic. Over half of all cord sera had the elevated levels of IgE (an immunoglobulin which does not cross the placenta), and more than 1/3 contained filariaspecific IgE antibody (determined by RAST). These findings are unique and indicate that prenatal sensitization to filaria does occur and probably affects subsequent responses to parasite infection (Weil, Hussain, Ottesen).

Variable patterns on immune recognition and response to parasite antigens among different individuals clearly exist and may also be important in determining both protective immunity and the different manifestations of human disease. Therefore, efforts to characterize the significant immunogens and allergens from the complex antigenic make-up of the parasite have been undertaken using a variety of immunochemical tools that allow fractionation of parasite antigens and subsequent analysis of the host response to them; eg. the technique of crossed radio immunelectrophoresis, CRIE (Hussain, Anwar, Ottesen; see annual report of Dr. Hussain).

In previous studies the prominence of immunoregulatory mechanisms in delayed hypersensitivity responses to chronic helminth infections has been emphasized. Since these infections also elicit high levels of specific IgE antibody (bound to mediator-rich basophils and mast cells) and since these cells are exposed in vivo, often continuously, to parasite antigens, we explored the reasons why allergic reactions (immediate hypersensitivity (IH) responses) are uncommon in chronic helminth infections. Our findings indicate that patients with chronic filariasis develop "blocking antibodies" in their sera which limit or contain IH responsiveness and that these antibodies are IgG directed against parasite antigens. Individuals who are least reactive to the parasite (ie. those with asymptomatic microfilaremia) have the highest titers of blocking antibody, while those with the least blocking antibody (relative to IgE antibody) are the ones with allergic symtomatology (ie. patients with TE) (Ottesen).

Additional studies of the mechanisms involved in the immunopathogenesis of filariasis have been carried out in animal models (Weil, Ottesen).

Schistosomiasis. Previous studies in our laboratory have defined the progressive loss of parasite antigen-specific cellular immune responsiveness in patients whose schistosome (Schistosoma mansoni) infections evolve from the acute to the chronic stages. This "loss" has been found actually to reflect the development of specific immunoregulatory mechanisms in these patients. Suppressive elements have been idetified (though not well characterized) both in the sera of chronically infected patients and among their adherent mononuclear cell populations derived from blood. Immediate hypersensitivity responsivness that is parasite specific reflects IgE antibody production has also been found (Ottesen, Hussain, Hofstetter). Modulation of immediate hypersensitivity has been shown to occur in these patients and to involve the development of schistosome specific IgG 'blocking antibodies', similar to those described in filariasis (Hofstetter, Ottesen). Other mechanisms for the immunosuppression of lymphocyte function in these patients (involving both T-lymphocytes and histamine) are currently under study. (Hofstetter, Ottesen).

The efficacy of mass treatment programs for schistosomiasis has been well established. However, the effect of treatment on patients' immune responses is still unknown. These changes may be of special importance when treated individuals are re-exposed to schistosomiasis or when future immunization programs are planned. Therefore, pre and post-treatment immunological studies have been carried out as part of a continuing collaborative research-treatment field project in Puerto Rico. Treatment of chronically infected patients with oxamniquine has resulted in a significant increase in cell mediated immune responses to parasite antigens. These enhanced responses have been associated with a reduction in the immunoregulatory mechanisms previously suppressing the responses, especially the adherent suppressive mononuclear cells. Also noted was a reduction in parasite specific IgE and IgG blocking antibody after successful therapy (Hofstetter, Ottesen).

S. mekongi is a newly recognized schistosome parasite of man. Twelve Laotian refugees with this infection have been evaluated at the NIH with respect to their clinical and immunologic responses to infection. This study is the first detailed examination of such patients; our findings suggest that many of those same generalization which have been made from studies of the host's clinical and immune responses to S. mansoni infection (both delayed and immediate hypersensitivity) apply to S. mekongi infections as well (Hofstetter, Barral, Nash, Cheever, Ottesen).

Additional studies of the mechanisms involved in the immunopathogenesis of schistosomiasis (both S. mansoni and S. japonicum) have been carried out in primate animal models (Barral, Cheever, Ottesen).

The Eosinophil. Eosinophils are prominent in the host response to allergens and helminth infections. These cells demonstrate autofluorescence of their cytoplasmic granules. With data derived from the spectral analysis of the fluorescence, a technique was devised to separate these cells from other leukocytes using the fluorescence-activated cell sorter. Sorted cells were highly purified, viable, and functional in vitro. The amount of fluorescence varied inversely with absolute eosinophil counts of the patients under study. The compound responsible for this fluorescence has been identified as flavine mononucleotide, a co-factor of certain oxidative enzymes which are especially prominent in the eosinophil (Weil, Chused).

Publications:

1. Catto, B.A. Schistosoma mansoni: Decarboxylation of 5-hydroxytryptophan, L-Dopa, and L-histidine in adult and larval schistosomes. Exp. Parasitol. 51, 152-157 (1981).

2. Catto, B.A., Lewis, F.A. and Ottesen, E.A.: Cercaria Induced Histamine Release: A Factor in the Pathogenesis of Schistosome Dermatitis? Am. J. Trop. Med. Hyg. 29, 886-889 (1980).

3. Hiatt, R.A., Ottesen, E.A., Sotomayor, Z.R. and Lawley, T.J.: Serial observations of circulating immune complexes in patients with acute schistosomiasis. J. Infect. Dis. 142, 665-670 (1980).

4. Weil, G.L., Ottesen, E.A. and Powers, K.G.: Dirofilaria immitis: Parasite specific humoral and cellular immune responses in experimentally infected dogs. Exp. Parasitol. 51, 80-86 (1981).

5. Weil, G.J. and Chused, T.M.: Eosinophil autofluorescence and its use in isolation and analysis of human eosinophils using microfluorometry. Blood 57, 1099-1104 (1981).

6. Weller, P.F., Ottesen, E.A. and Heck, L.: "Immediate and Delayed Hypersensitivity Skin Test Responses to the Dirofilaria immitis Filarial Skin Test (Sawada) Antigen in Wuchereria bancrofti Filariasis. Am. J. Trop. Med. Hyg. 29, 809-814 (1980).

7. Weller, P.F., Ottesen, E.A. and Goetzl, E.J.: "Sequential Alterations in the Human Eosinophil Content of Arylsulfatase B During Therapy of Bancroftian Filariasis." Clin. Immunol. and Immunopathol. 18, 76-84 (1981).

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14. Ackerman, S.J., Gleich, G.J., Weller, P.F. and Ottesen, E.A.: Eosinophilia and elevated serum levels of eosinophil major basic protein and Charcot-Leyden crystal protein (lysophospholipase) following treatment of patients with Bancroft's filariasis. J. Immunol. (in press).

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17. Gusmao, R. d'A, Stanley, A.M. and Ottesen, E.A. Brugia pahangi: Immunologic evaluation of the differential susceptibility to filarial infection in inbred Lewis rats. Exp. Parasitol. (in press).

18. Hamilton, R.G., Hussain, R., Ottesen, E.A. and Adkinson, N.F.jr., The quantitation of parasite-specific human IgG and IgE in sera: evaluation of solid phase RIA and ELISA methodology. J. Immunol. Meth. (in press).

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20. Weiss, N., Hussian, R. and Ottesen, E.A. IgE antibodies are more species-specific than IgG antibodies in human onchocerciasis and lymphatic filariasis. *Immunol.* (in press).

21. Barral-Netto, M., Hofstetter, M., dosSantos, J.G., Cheever, A.W. and Ottesen, E.A. *Schistosoma mekongi* infection in man: Cellular immune responses and modulating mechanisms. *Clin. Exp. Immunol.* (in press).

22. Hofstetter, M., Nash, T.E., Cheever, A.W., dos Santos, J.G. and Ottesen, E.A. Schistosomiasis mekongi in Southeast Asian Refugees, *J. Inf. Dis.* (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 0094-22 LPD

PERIOD COVERED
September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)
Studies on Entamoeba histolytica and other Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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L. Jacobs	Scientist Emeritus, NIH
D.B. Keister	Biologist, LPD, NIAID
E.C. Weinbach	Head, Physiology and Biochemistry Section, LPD, NIAID
C.F.T. Mattern	Medical Officer, LPD, NIAID
V. Gill	CC,CP

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.3	2.7

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A partially defined medium, 294 devised for axenic cultivation of Entamoeba histolytica facilitates study of nucleic acid metabolism and furthers investigation of nutritional requirements of the parasite. An improved medium, TYSGM-9 has been devised for xenic cultivation of lumen dwelling protozoa inhabiting the alimentary tract of man. The bile factor stimulating growth of Giardia lamblia in vitro has been shown to be a low molecular weight compound. Development of a new cryopreservent permits freeze-preservation by controlled rate and uncontrolled slow freezing of 14 species of lumen dwelling protozoa (Entamoeba. Dientamoeba, Giardia, Trichomonas, and Crithidia), Medium 114, a partially defined medium in which the carbohydrate content can be controlled precisely, has been used to study encystment of the laboratory model Entamoeba invadens under axenic conditions, and to facilitate study of the effects of specific bacterial extracts and metabolites on encystment of axenized E. histolytica.

PHS-6040
(Rev. 10-76)

Project Description:

The objectives are four-fold: 1) to develope and refine techniques for cultivation of lumen dwelling protozoa, especially Entamoeba histolytica and Giardia lamblia; 2) to determine their nutritional requirements in axenic in vitro systems and to define the physical and chemical conditions for optimal growth; 3) to study the mechanisms of their pathogenicity; 4) to devise or improve existing methods for freeze-preservation of these parasites at cryogenic temperatures and to study mechanisms involved in resistance to freezing injury.

A. Developement of a Defined Medium for Axenic Cultivation of Entamoeba histolytica. - TYI-S-33 the medium currently used for axenic cultivation of E. histolytica contains three undefined biological products, Trypticase a caesin digest, yeast extract (YE) and bovine serum. In an earlier report we noted that YE had been replaced with a mixture of defined nutrients, i.e. adenine, guanine, cytosine, uracil, adenine nucleotides, ammonium chloride and B vitamins (Medium 114). Current studies are aimed at defining the essential bases and nucleotides, and the study of the corresponding nucleosides as nutrilites.

5' adenine nucleotides found in yeast were not required for amebic growth in the presence of the four bases. However, they were essential when the nucleosides of the bases were substituted. Mixtures of 5' nucleotides, i.e. AMP, GMP, CMP, and UMP, could not substitute for the bases. Adenine alone, of all the compounds tested, could support serial subculture of the amebae. However, growth was below that obtained with a mixture of the four bases. As an outgrowth of these studies a simpler and more sensitive medium, 294, was developed to further our investigations of YE replacement.

Future Investigations: Our efforts will be directed toward replacing the Trypticase and serum components of TYI-S-33 with defined nutrients. Selected compounds added to MM-1, a defined short term maintainence medium (Gillin and Diamond), will be studied for their ability to stimulate amebic mutiplication.

B. Axenic Cultivation of Giardia lamblia. - TYI-S-33 medium supplemented with bile has proved superior to previously described media for cultivation of G. lamblia. Preliminary study of the bile supplement using dialysis and ultrafiltration shows the growth stimulating factor (s) has a molecular weight between 1000 and 10,000.

Future Investigations: Attempts will be made to identify the factor (s) by chromatographic fractionation. (Keister).

C. New Medium for Xenic Cultivation of Lumen Dwelling Protozoa. - TYSGM-9 medium, devised to facilitate the transition of E. histolytica from xenic to axenic growth has proved highly efficient for initiation and maintenance of cultures of the following protozoa inhabiting the intestine or mouth of man: E. histolytica, E. coli, E. gingivalis, Dientamoeba fragilis, Trichomonas tenax, and Balantidium coli. Its utility in cultivation of other species from these sites has not been tested. TYSGM-9 offers distinct advantages over previously devised media. Its clarity permits monitoring the protozoa in situ, growth is consistently more luxurious and it is easily prepared from ingredients commonly available in diagnostic microbiological laboratories.

D. Studies on Encystment of E. histolytica Under Axenic Culture Conditions. Entamoeba histolytica does not encyst under axenic conditions. In sharp contrast, amebae grown xenically with bacteria (A-B cultures) encyst more or less regularly and can be induced to encyst en masse by the following method. The amebae are starved by depriving them of carbohydrate. Addition of carbohydrate initiates intense multiplication which is followed by mass encystation. Using the partially defined medium 114 in which carbohydrate content can be controlled precisely, attempts were made to reproduce the carbohydrate starvation-repletion cycle under axenic conditions. Failure to obtain cysts suggested involvement of other mechanisms in the A-B cultures.

The role of bacteria in cyst production in A-B cultures has not been elucidated. Availability of axenic cultures and development of medium 114 offer unique opportunities to investigate this relationship. Furthermore, the components of a fecal bacterial flora (NRS), characterized by its ability to induce encystation of E. histolytica. have been identified to the level of species (collaborative efforts with Dr. Vee Gill, CC/CP).

Future Investigations: The NRS isolates will be examined singly and in combination for their ability to induce encystation. Living bacteria, bacterial extracts and metabolic products will be studied. During the past year medium 114 has been used to induce encystation of the reptilian pathogen E. invadens more efficiently than previously described media. This will be the basis for reexamining encystment of axenized E. invadens as a model for similar differentiation of E. histolytica. (Diamond, Keister, Jacobs).

E. Cryopreservation of Lumen Dwelling Protozoa. - Frequently, a technique devised for cryopreservation of a protozoa cannot be applied to culture variants, strains of the same species, or other species. Development of a new cryopreservant consisting of DMSO, sucrose, serum and culture medium, has permitted controlled-rate freezing, and with minor modifications of ingredient concentrations, uncontrolled slow freezing of all lumen dwelling protozoa in our laboratory collection. This includes 14 species representing 5 genera, Entamoeba, Dientamoeba, Trichomonas, Giardia, and Crithidia, all strains and all culture variants. Organisms recovered after freezing provide functional cultures within one week of thawing.

Future Investigations: In view of the accomplishments made in freeze-preseration of lumen dwelling protozoa only minor efforts will be made to improve the present technology. Major emphasis will be given to the study of the isoenzyme patterns of selected protozoa in our collection and at the ATCC. (Diamond, Keister, Daggett, Gillin).

F. Significance to Biomedical Research and the Program of the Institute. Media developed for axenic cultivation of E. histolytica and G. lamblia, two important intestinal pathogens of man, provide means of obtaining large numbers of bacteria-free organisms for antigen production, biochemical, physiological, immunological and pathological investigations. A medium devised for one parasite frequently can be employed for an unrelated species. TYI-S-33 devised for cultivation of E. histolytica has proved more efficient for growing Trichomonas vaginalis than media designed specifically for the purpose (C F.T. Mattern, LPD). Defined media are prerequisite for elucidation of the nutritional requirements of parasites and facilitate study of membrane biolipids.

The principle advantage of cryopreservation is its ability to halt continuous reproduction of an organism as it occurs in culture or animal passage and thus prevents continuous selection and/or mutation. The techniques developed in this laboratory enable the preservation of a wide spectrum of lumen dwelling protozoa.

Infection of *E. histolytica* and *G. lamblia* are transmitted from host to host by means of the cyst stage. Ability to produce this stage in axenic cultures would permit study of this important aspect of parasite differentiation under precisely controlled conditions.

For results of collaborative studies with investigators in other sections within the Laboratory of Parasitic Diseases see the following annual reports: Project No. ZO1 AI 0098-25, PI E.C. Weinbach; Project No. ZO1 AI 00185-03, PI C.F.T. Mattern.

Publications:

1. Diamond, L.S.: Axenic cultivation of *Entamoeba histolytica*: Progress and Problems. Arch. Invest. Med. (Mex.). 11 (Supl 1): 47-54, 1980.

2. Diamond, L.S.: A new liquid medium for xenic cultivation of *Entamoeba histolytica* and other lumen dwelling protozoa. J. Parasitol. (in press).

3. Gillin, F.D. and Diamond, L.S.: Attachment and short-term maintenance of motility and viabiltiy of *Entamoeba histolytica* in a defined medium. J. Protozool. 27: 220-225, 1980.

4. Gillin, F.D. and Diamond, L.S.: *Entamoeba histolytica* and *Giardia lamblia*: Growth responses to reducing agents. Exper. Parasitol. 51: 382-391, 1981.

5. Gillin, F.D. and Diamond, L.S.: Inhibition of clonal growth of *Giardia lamblia* and *Entamoeba histolytica* by metronidazole, quinacrine, and antimicrobial agents. J. Antimicrobial Chemother. (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00097-23 LPD

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Physiological and Cytochemical Pathology of Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Dr. John J. Pisano	Chief, Physiological Chemistry Section	LC, NHBLI
Drs. Victor Ferrans and J. Butany	Pathologists	PB, NHBLI
Dr. H. Donald Hochstein		DCA, BOB

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Laboratory of Chemistry and Pathology Branch, NHLBI
Bureau of Biologics

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Laboratory of Parasitic Diseases

SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12/12	12/12	0

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SUMMARY OF WORK (200 words or less - underline keywords)

An anti-trypanosomal factor (ATF) produced by Pseudomonas fluorescens against the Tulahuen strain of Trypanosoma cruzi consisted of two main fractions: ATF-I, exhibited a molecular weight higher than 20,000, was less active against the flagellates and produced toxic effects in mice. ATF-II, of molecular weight approximately 1,000, was quite active against the parasites, and well tolerated in mice. In view of the protective effects observed in vivo and of the potential significance of these findings on the chemotherapy of Chagas' disease further studies on the chemical characterization of the ATF and on its pharmacological and toxicological properties will be made.

PHS-6040
(Rev. 10-76)

Project Description: Further studies on the purification of the antitrypanosome factor (ATF) were made employing Sephadex gel filtration and polyacrylamide gel electrophoresis. The Limulus amoebocyte lysate test was used to detect the presence of lipopolysaccharide (LPS). Hemolytic and proteolytic properties were detected according to standard techniques. Electron microscopic observations of the flagellates were made following exposure to 1 mg of ATF-II protein for various intervals of time. In vivo studies were performed employing intramuscular injections of ATF-II in mice following intraperitoneal inoculations of 1,000 parasites.
Two main fractions were recovered from the crude samples. ATF-I, exhibited a molecular weight larger than 20,000. ATF-II was considerably smaller (molecular weight approximately 1,000). Both fractions were active against the flagellates but the activity of ATF-I was distinctly lower. In preliminary trials with mice, ATF-I was observed to be toxic and some animals died within 48 hours. Polyacrylamide gel electrophoresis revealed three main bands in the ATF-I; the most anodic and cathodic bands stained positively for carbohydrate. ATF-II exhibited one main band and did not contain polysaccharide. The first fraction was separated with dialysis of the crude samples employing tubing of 6-8,000 molecular weight cut-off. A large proportion of the ATF-II was also recovered with dialysis employing tubing of 1,000 molecular weight cut-off and/or by desalting with Sephadex G-10. The main variation observed among the crude samples was related to the total protein yield which was attributed at least to some extent to methodology. Total protein levels of 20 to 40 milligrams were obtained (based on 200 ml of the crude sample), and often a recovery of 70 to 80 percent in the active fractions. No hemolysis was produced by either fraction and they were not proteolytic. ATF-I contained 10 times more LPS than ATF-II however, the presence of LPS did not appear to be related to the lytic effect on the parasites since fraction I which contained the higher amount exhibited a much lower activity in our assays against the parasites. In addition supernatant fluids obtained after sonication of the bacterial cells did not enhance the lytic effect of the factor secreted extracellularly.

ATF-II-treated flagellates revealed: edema of the cytoplasm; edema, swelling, and disruption of the kinetoplast and mitochondrion; appearance of clear vacuoles, dilated tubules, and lamellar structures in the cytoplasm; clumping of nuclear chromatin, and focal disruption of plasma membranes. These changes were considered of progressive, eventual lethal injury.

In vivo studies indicated a protective effect againt the progression of the blood infection in 20-gram male mice which received a total of 6 grams of the ATF-II protein over a period of 58 days. Microscopic observation of liver and spleen from animals killed after various intervals of treatment indicated that although parasites were able to penetrate and multiply, since some organisms and histological changes were observed 13 days after inoculation, the usual progression of the infection was controlled leading to the disappearance of the trypomastigotes from the blood stream (30 negative microscopic fields) and restoration of normal tissue architecture. All untreated infected animals and other untreated infected which were injected with buffer instead of the ATF died 13 to 21 days after inoculation.

Proposed Course of the Project: Studies on the chemical characterization of the ATF and its effects on the ultrastructure of the parasites and of parasitized tissues will be continued in collaboration with specialists in the National Heart, Blood, and Lung Institute.
Attempts already initiated to produce antibodies in rabbits against the ATF-II will be continued. This would aid greatly in label studies, e.g., in identifying the molecule within the tissues and/or in determining the duration of its activity in the blood stream.
The protective effects observed in vivo should be examined further under more controlled conditions such as in tissue culture.
The progress made so far on this project has been possible because of the excellent collaboration of interested specialists in related fields. It seems however, that future accomplishments will depend greatly an the availability of technical assistance assigned to the principal investigator who until now has been soley responsible not only for the basic laboratory work but for the direction and development of these studies.

The observed relationship between a trypanosome and a bacterium provides another interesting example of microbial interaction. The ATF effects on the ultrastructure of the parasites, which were more extensive than those reported previously for Lampit, the most effective drug used today in controlling Chagas' disease, were encouraging in view of the need for more effective chemotherapy of this important parasitic disease.

Publications:

Mercado, T.I. Studies on the pathophysiology of Trypanosoma cruzi. (Symposium paper presented at the SACNAS Conference, November, 1980) Proceedings of the Conference. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00098-25 LPD

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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S. Wieder	Medical Technologist, LPD, NIAID
D.B. Keister	Research Microbiologist, LPD, NIAID

COOPERATING UNITS (if any)

Laboratory of Chemical Physics, NIADDK (H. Kon)
Clinical Neuropharmacology Branch, NIMH (J.L. Costa)
Metabolism Branch, NCI (D. Tschudy and P. Ebert)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Physiology and Biochemistry

INSTITUTE AND LOCATION

NIAID, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of aerobic energy metabolism were continued to elucidate mechanisms of electron transfer in the respiratory chains of Entamoeba histolytica and Giardia lamblia. Purification and preliminary characterization of the oxidoreductases in these parasites was done by affinity chromatography and gel electrophoresis. Specific activity of the NAD(P)H oxidoreductases of G. lamblia was increased 50-fold, and separation of the enzymatically active proteins by gel electrophoresis yielded 6 bands. Flavin was present in a 1:1 molar ratio to protein. These enzymes provide a mechanism for oxidation of NADH generated during glycolysis since both mitochondria and lactate dehydrogenase are absent in these eukaryatic protozoans. Studies were continued on the effect of heme depletion on the respiration of cultured murine erythroleukemia cells and isolated rat hepatocytes.

Mammalian studies were initiated on the effect of psychoactive drugs on oxidative phosphorylation in mitochondria isolated from rat brain and liver. Reserpine acts as a classical uncoupling agent only when dissolved in organic solvents to facilitate penetration through the mitochondrial membranes. Pargyline and the biogenic amines do not affect the four states of mitochondrial respiration.

Project Description:

The object of this project is to conduct fundamental studies on mechanisms of aerobic energy metabolism in parasites and mammalian cells. Currently, these studies center primarily on identification and characterization of components of the respiratory chains of pathogenic, enteric protozoa, and elucidation of the pathway of electron flow to molecular oxygen. Purification of oxidoreductases are a vital part of this program. Other (collaborative) studies focus on bioenergetic phenomena in organelles from flagellates, mammalian tissues and cultured tumor cells.

Tissue and cells are disrupted mechanically and subcellular fractions isolated by differentia and gradient centrifugations. Cellular and subcellular constituents, enzyme activities, and metabolic pathways are determined, characterized and elucidated by chemical, radiochemical, physical and enzymatic methods. Changes in oxygen concentrations are determined polarographically with the Clark electrode. Electron paramagnetic and nuclear magnetic resonance spectrophotometry is used to determine non-heme iron proteins and ^{13}C-labeled metabolites.

Parasite Studies:

Continued investigation of electron transfer in *E. histolytica* and *G. lamblia* have revealed marked differences in the subcellular localization and properties of the respiratory chains of these intestinal parasites. The bulk of respiratory enzymes and electron carriers were found in the cytosol of *E. histolytica*, and in the particulate fraction of *G. lamblia*. The cytosol of *G. lamblia* also contain highly active oxidoreductases which have been purified 50-fold by affinity chromatography utilizing dicumarol liganded to sepharose. Characterization of the purified enzymes by gel electrophoresis yielded six bands with a 1:1 molar ratio of flavin to protein. The soluble oxidoreductase of both enteric protozoa resemble the mammalian "DT-diaphorase" in their sensitivity to inhibitors and reactivity with exogenous electron carriers (Weinbach, Weider, Claggett). In collaboration with Dr. J.L. Costa, NIMH, the technique of nuclear magnetic resonance (NMR) has been used to identify the metabolites formed from carbon-13 labeled glucose in trophozoites of intact *G. lamblia*. The preliminary results illustrate the potential advantage of the NMR method for a non-destructive, highly-specific analysis of all metabolites from labeled substrates in studies of parasite biochemistry (Weinbach, Weider and Costa). Investigation of L-serine oxidation in *E. histolytica* continued to reveal baffling intricacies of this reaction. Studies this year have shown that the current strain of amebae when serially cultivated in the old TP-S-1 medium gradually exhibits pyruvate oxidation in the presence of CoA, but only feeble oxidation of L-serine. Thus, serine dehydratase, is ~~not~~ restored under these conditions, whereas pyruvate oxidase is (Weinbach, Keister).

Mammalian studies:

In collaboration with J.M. Costa, Staff Physician, NIMH, a study of the effects of various neuropharmacological agents on oxidative phosphorylation and protein flux in mammalian mitochondria was initiated. Resperpine in micromolar amounts acts as a classical uncoupling agent. However, this action is observed only if the reserpine is dissolved in organic solvents or is depronated; conditions necessary for the drug to penetrate the mitochondrial membranes. This conclusion is supported by the observation that reserpine in water uncouples oxidative phosphorylation in isolated mitoplasts (mitochondria free of outer membranes). Pargyline, and the biogenic amines benzylamine and phenethlamine, in contrast, do not affect any of the four states of mitochondrial respiration. Mitochondria from rats injected with pharmacologic doses of pargyline or reserpine show no respiratory alterations (Weinbach, Claggett and Costa). Collaboration with Dr. Tschudy, NCI, was continued on the effect of heme depletion on respiratory activity of cultured murine erythroleukemia cells. Only after drastic reduction of heme synthesis by exposure of the cells to 4, 6-dioxoheptanoic acid were perturbations in respiratory activities observed, indicating the remarkable preservation of cytochromes in these tumor cells, as was observed last year in isolated rat hepatocytes (Weinbach, Tschudy and Ebert).

This project, which attempts to elucidate vital biochemical mechanisms common to all aerobic cells, has obvious implications for biomedical research in general (illustrated by the collaborative projects), and provides a rational basis for understanding bioenergetic mechanisms associated with parasites and parasitism. The parasite studies will be continued, again with the emphasis on identifying the ultimate electron carrier in the enteric protozoans that replaces cytochromes found in other eukaryotes. The most likely candidate is an non-heme iron sulfur protein in view of our recent experiments with high potential electron donors. Molecular characterization of the NAD(P)H oxidoreductase of G. lamblia will be done as soon as the improved method for cultivating large amounts of this parasite have been completed. In collaboration with Dr. P. Smith, we will attempt to identify the protein constituents of G. lamblia tropozoites which are chemotactic for human monocytes and granlocytes. Mammalian studies of psychoactive drug effects on isolated mitochondria will be extended to human platelets. Studies of heme depletion and respiratory metabolism of cultured tumor cell will be continued. Studies of Ca modulation of ATPase activities and Ca transport by calmodulin in G. lamblia will be extended to L. donovani in collaboration with Dr. Dwyer.

Publications:

Weinbach, E.C., Takeuchi, T., Claggett, C.E., Inoue, F., Kon, H., and Diamond, L.S.: Role of iron-sulfur proteins in electron transport system of Entaomeba histolytica. Arch. Invest. Med. (Mex) 11 (Supl.1) 75-81 (1980).

Weinbach, E.C., Claggett, C.E., Keister, D.B.,Diamond, L.S. and Kon, H.: Respiratory metabolism of Giardia lamblia. J. Parasitol. 66: 347-350 (1980).

Tschudy, D.P., Ebert, P.S., Hess, R.A., Frykholm, B.C. and Weinbach, E.C.: Effect of heme depletion on growth, protein synthesis and respiration of murine erythroleukemia cells. Biochem. Pharmacol. 29: 1825-1831 (1980).

Pazoles, C.J., Weinbach, E.C., Creutz, C.E. and Pollard, H.B. Inhibition of ATP/anion osmotic lysis of chromaffin granules by mitochondrial uncouplers and its relevance to secretion by exocytosis. J. Supramolecular Biol. (in press).

Weinbach, E.C. Biochemistry of enteric parasitic protozoa. Trends in Biochemical Sciences (in press).

PROJECT NUMBER (Do NOT use this space)	HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	Z01 AI 00099-11 LPD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biophysical Parasitology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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V. Bongertz	Visiting Fellow, LPD, NIAID
M. Postan	WHO Fellow
J.C. Engel	WHO Fellow

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Instituto "Fatala Chaben", Buenos Aires, Argentina
London School of Hygiene and Tropical Medicine, London, UK

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
60/12	51/12	9/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Flow cytometric analysis of the total DNA/parasite of 31 clones and 2 strains of Trypanosoma cruzi demonstrates stable differences between populations about 30%. The G1, S and G2 fractions of the various clones have been correlated to their growth rate. The infection of vertebrate cells by T. cruzi and Toxoplasma gondi has been shown to be modulated by the vertebrate cell cycle implying that receptor(s) are required for this interaction and qualitative or quantitative changes in the level of these receptor(s) occur. T.cruzi clones isolated from chronic chagasic patients exhibit both inter- and intra-patient differences in their growth rates. A clone of T.cruzi has been isolated which grows only as extracellular amastigotes under routine LIT culture conditions. Both clone-specific and strain-specific T.cruzi antigens have been demonstrated to occur. Some of these antigens may correlate to disease state. The susceptibility of inbred strains of mice and the course of disease varies with the clone of the parasite. In addition, both qualitative and quantitative differences in morbidity and mortality and histopathology occur in mice infected with T.cruzi clones derived from the same source.

PHS-6040
(Rev. 10-76)

Project Description

An interdisciplinary approach is being utilized to study the interaction of Trypanosoma cruzi with vertebrate cells and the basic cell biology/physiology of T. cruzi The shift in emphasis from T. cruzi "strains" to the use of single cell-isolate T. cruzi clones described in the previous report has allowed more precise and unambiguous studies and elucidation of several aspects of the biology of the cycle of T. cruzi. Five major topics are being studied: 1. Flow cytometric analysis of T. cruzi clones; 2. Characterization of receptors involved in T. cruzi and Toxoplasma gondii-vertebrate cell interactions; 3. Cell biology of T. cruzi clones isolated from chronic chagasic patients in Argentina; 4. Immunochemical analysis of T. cruzi clones; and 5. Immunopathology and parasitology of the infection of inbred strains of mice with T.cruzi clones.

1. Flow Cytometric Analysis of T. cruzi Clones

A flow cytometer/florescence-activated cell sorter (EPICS II) was obtained from the National Cancer Institute. Following extensive modification and testing, the instrument has been used for studies of the DNA profile of T.cruzi clones. Total DNA/cell is quantified as the amplitude of fluorescence emission folowing specific staining with mithramycin and irradiation at 457 nm. The G1, S and G2 subpopulations of T. cruzi epimastigote cultures in both the exponential ans stationary phase of growth have been identified and quantified. Analysis of total DNA/parasite has demonstrated that large differences exist between T. cruzi clones. Thus far, 30% differences in the total DNA/parasite have been found between clones. The absolute DNA values are stable. It has also been shown that there is a dispersion in DNA values of clones isolated from a single stock which underscores the concept of heterogenity of T. cruzi stocks or "strains".

The proportions of the parasite population in the G1, S and G2 stages of the cell cycle have been correlated to growth rate. The G1 fraction of the population is inversely proportional to the log of the growth rate, the S fraction is directly proportional to the log of the growth rate and the G2 fraction is directly proportional to the growth rate.

2. Characterization of Receptors Involved in T. cruzi and T. gondii-Vertebrate Cell Interactions.

Vertebrate cells (HeLa) were cell cycle synchronized by mitotic shakeoff. These cells were exposed at 3 hr. intervals for 24 hrs. to either T. cruzi or T. gondii in the presence of ^{3}H-thymidine and processed for autoradiography. The mean number of parasites/cell, the percent infected cells, the percent labeled nuclei and mitotic index were determined at each exposure interval. The ability of both T. cruzi and T. gondii to infect HeLa cells increased as the HeLa cells traversed from the G1 to S phase of their cell cycle and decreased as the HeLa cells entered G2-M phase. This is the first demonstration that the infection of vertebrate cells by these parasites is modulated by the vertebrate cell cycle. It is postulated that cell surface component(s) acting as receptors are responsible for this phenomenon.

3. Cell Biology of T. cruzi Clones Isolated From Chronic Chagasic Patients in Argentina.

Nineteen single-cell isolate clones of T. cruzi were obtained from insects fed upon 2 patients with chronic Chagas' disease. The growth rates in LIT medium of the individual clones were determined. The doubling times of the clones were distributed into 2 groups. One group consists of parasites with a mean doubling time of about 24 hrs. and the other group consists of parasites with a mean doubling time of about 50 hrs. The doubling times are stable. However, not all of the clones isolated from the same patient exhibit the same population doubling time. This is the first demonstration of heterogeniety in the growth rate of T. cruzi subpopulations present in a human infection. In addition, one of the clones reproduces as amastigotes extra-cellularly under routine culture conditions in LIT medium. These amastigotes are resistant to complement mediated lysis as are amastigotes liberated from infected cells. All of the clones are capable of infecting vertebrate cells in culture. The kinetics of growth in vertebrate cells parallels the results obtained in LIT medium. That is, clones which grow slowly in LIT medium also grow slowly in vertebrate cells.

4. Immunochemical Analysis of T. cruzi Clones

Whole homogenates of 6 T. cruzi clones and 2 T. cruzi strains were injected into rabbits. The rabbits developed antibodies against this material. These antibodies are being characterized using a variety of immunochemical methods. Between 15 and 30 antigen-antibody lines per clone or stock have been identified and classified. Thus far, it has been shown that, as expected, common antigens exist between these populations. However, both clone-specific as well as stock or "strain"-specific antigens have also been demonstrated. Some of these differences appear to correlate to the disease state (i.e. chronic or acute) of the patient from which the clones were isolated.

5. Immunopathology and Parasitology of the Infection of Inbred Strains of Mice with T. cruzi Clones.

There are many conflicting reports concerning the course of infection of inbred strains of mice with T. cruzi strains. As laboratory strains of the parasite may represent highly selected subpopulations and recently isolated strains may represent heterogeneous populations, this study utilizes clones of T. cruzi which represent a homogeneous and stable genetic background. It has been demonstrated that the susceptibility of mouse strains and course of disease varies depending upon the clone of the parasite used. Contrary to popular belief, C3H mice are not uniformly more susceptible than C57 mice to infection by T. cruzi. The obverse situation has been demonstrated using T. cruzi clones. Qualitative and quantitative differences in morbidity, mortality and histopathology have also been shown to occur in mice infected with T. cruzi clones derived from the same strain. This extends the findings of the biological diversity of T. cruzi clones and demonstrates that this diversity is also relevant to the course of the infection of intact animals.

Significance and Proposed Course of Program:

The study of host-parasite interactions at the cellular and subcellular levels is a topic of critical importance. The outcome of this interaction directly influences the physiologic state and survival potential of both the host and the parasite.

Flow cytometric analysis of T. cruzi clones will be continued in an attempt to elucidate the basis (i.e. kinetoplast or nucleus) for the large differences in the total DNA/parasite which occur. In addition, flow cytometric analysis of the DNA profile of parasites other than T. cruzi will be started. The flow cytometer will be modified to allow adsorption studies. In conjunction with color-yeilding biochemical reactions for respiratory enzymes, this should allow correlative studies between cell cycle and isoenzyme expression. Vertebrate cell and T. cruzi surface membranes will be prepared in an attempt to isolate and characterize the cell cycle-related component(s) responsible for modulating the infection of vertebrate cells by the parasite. The intracellular cycle of the 19 T. cruzi clones isolated from chronic chagasic patients will be quantified and the amastigote clone will be further characterized. The study will be expanded to include clones derived from humans with acute T. cruzi infections. Florescent-labeled antibodies will be prepared in order to determine if clone, "strain", cell cycle and stage-specificity of the immune response exists. Quantification will be by flow cytometry. Studies of the course of the infection of inbred strains of mice with T. cruzi clones will be amplified to include histochemical muscle typing and immune responses.

Publications:

1. Dvorak, J.A.: Trends in the use of in vitro cell cultures for Trypanosoma cruzi research. in The in vitro Cultivation of the Pathogens of Tropical Diseases. Tropical Diseases Research Series 3. pp 223-236. D. S. Rowe and H. Hirumi (eds) Schwabe & Co., Basel 1980.

2. Crane, M.S. and Dvorak, J.A.: Trypanosoma cruzi: Interaction with vertebrate cells.Pattern of RNA synthesis following infection of vertebrate cells. J. Protozool. 27: 336-338, 1980

3. Dvorak, J.A., Hartman, D.L., and Miles, M.A.: Trypanosoma cruzi: Correlation of growth kinetics to enzyme type in clones derived from various sources. J. Protozool. 27: 472-474, 1980.

4. Garcia, E.S. and Dvorak, J.A.: Growth and development of two Trypanosoma cruzi clones in the insect Dipetalogaster maximus. Am. J. Trop. Med. Hyg. (in press).

5. Postan, M., McDaniel, J.P. and Dvorak, J.A.: Estudio anatomopatologico comparativo de ratones infectados con clones de Tripanosoma cruzi. in Proceedings of the XIII Latinamerican Congress of Pathology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00102-07 LPD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Disease Caused by Infection with Intracellular Parasites.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F.A. Neva	Chief, Laboratory of Parasitic Diseases, NIAID
Others:	Eskild Petersen	Collaborative Researcher (IPA), LPD,NIAID
	Robert Genta	Collaborative Researcher (IPA), LPD,NIAID
	Albert Gam	Biological Laboratory Technician (Micro.),LPD,NIAID
	Renato Gusmão	Guest Investigator, LPD,NIAID
	R. Corsey	Electrical Engineer, Applied Clinical Section, BEIB,NIH
	A. Barral	Visiting Fellow, LPD,NIAID
	J.D. Malley	Res.Mathematician, Div. Computer Research and Technology, LSM
	Eric A.Ottesen	Senior Invesitgator, LPD,NIAID

COOPERATING UNITS (if any) Dr. Humberto Bogaert, Institute of Dermatology, Dominican Republic. Dr. Peter Walzer, University of Kentucky Medical Center, Lexington, Kentucky. Dr. Murray Witner, Albert Einstein Medical School, New York. Dr. Francis Ward, Duke University Medical Center, Durham, North Carolina. (Contd.next page)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Cell Biology and Immunology Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
21/12	12/12	33/12

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Follow-up studies were conducted on the diffuse cutaneous leishmaniasis (DCL) from the Dominican Republic. Those with positive leishmanin skin tests in the endemic area generally exhibited lymphocyte proliferation to leishmanial antigen, in contrast to patients with DCL. Further observations on local heat treatment were made on one new DCL case and a previous drug-treated patient who relapsed. A number of leishmanial isolates from patients with known clinical features, known geographic origin and known virulence for the genetically susceptible BABL/c mouse are now available to correlate with results of biochemical taxonomy. The leishmanial isolates from the DCL cases are distinct from Western hemisphere species and appear to be a new group. ELISA and IHA serologic tests using antigens from cultured filariform larvae were developed for serodiagnosis of human strongyloides infection. Some of the findings on immune response in rats to experimental infections with S. ratti were applied to patients found infected with S. stercoralis. Lymphocyte proliferation to larval antigen was not found to be a useful or reliable criterion of infection. However, histamine release from IgE sensitized peripheral basophils after exposure to larval antigens was found to be a consistent indicator of human infection. Nature of the antigen causing histamine release is under study.

Cooperating Units - contd.: R. Correa-Coronas, National Naval Medical Center, Division of Infectious Diseases, Department of Medicine. Drs. Joffre Rezende and Anis Rassi. Federal University of Goias, Goiania, Brazil.

Project Description:

The Chagas' disease and T. cruzi work has been primarily analysis of past results with no new initiatives, as outlined below. There has been continuation of the studies with another intracellular parasite, the leishmania. This project has involved follow-up of the patients from the Dominican Republic with the unusual variety of cutaneous leishmaniasis of the diffuse variety (DCL) characterized by anergy to parasite antigen, multiple nodular rather than ulcerative lesions, chronicity and lack of response to usual chemotherapy. The approach to this project has been assessment of immunologic status of the patients, recovery and characterization of the causative strain of parasite and treatment of the disease. We have also continued work on assessing serologic tests for diagnosis of Strongyloides infection. Although this project involves a helminth and is somewhat outside the scope of our main interest, the work relates to parasitic infections of Southeast Asian refugees who have been referred to us, and it also fits into the work of an IPA investigator working with us.

1. Studies on Leishmaniasis. The four patients with DCL who were studied and treated at NIH were followed-up at 6 months and 1 year in the Dominican Republic. In addition, lymphocyte blastogenesis to leishmanial antigens was tested in leishmanin skin test positive as well as skin test negative individuals living in the endemic area of the country. Although there was considerable variation in response, the skin test positive cases were generally responsive, in contrast to patients with DCL. The treated cases of DCL had developed some reactivity to leishmanial antigens. (Petersen, A. Barral and R. Correa-Coronas of Naval Med.Center). One new case of DCL and an amphotericin-treated patient who had relapsed were brought to the NIH for local heat treatment. On the basis of cultures from pre- and post-treatment lesion biopsies, as well as clinical observations, the immediate results of heat treatment appeared to be favorable. (Neva and Corsey). Leishmanial isolates from several additional cases of cutaneous leishmaniasis have been obtained so that we now have at least 8 strains which can be serotyped to correlate biochemical taxonomy with virulence for the genetically susceptible BALB/c mouse, along with the clinical picture which the parasite produced in the patient. Already it is known that the parasites from the DCL patients are clearly different from the L. mexicana and L. braziliensis complexes, and may constitute a new species of leishmania. (Neva).

2. Studies on Strongyloidiasis. Extracts of filariform larvae of Strongyloides stercoralis or S. ratti, grown in charcoal cultures, were found to be almost interchangeable as suitable antigens in the ELISA test for detection of IgG antibodies in sera of patients infected with S. stercoralis. About 80-85 percent of parasitologically proven cases had significant levels of antibody. Only a few cases were negatide to one or

the other antigen. An antigen from larvae of a third nematode, Nippostrongylus braziliensis was unsuitable as an antigen. The same antigens did not react in a bentonite flocculation test, but did react reasonably well in an indirect hemagglutination test. (Neva and Gam). Humoral and cellular immune responses were studied in rats infected with large (3000) and small (100) inocula of S. ratti larvae. Rats given the large inoculum exhibited lymphocyte proliferative responses to larval antigen by mesenteric and peripheral lymphocytes, but a decline to near pre-infection levels soon thereafter. Serum antibody levels similarly declined 4 to 5 months after infection. Peripheral lymphocytes from patients with strongyloides infection show no predictable pattern of response and blastogenic responses have been observed in normal controls. (Genta). In addition to presence of serum antibodies to larval antigen by ELISA or IHA tests, the most reliable indicator of human infection with S. stercoralis is the histamine release reaction that can be shown when peripheral leukocytes (basophils) are exposed to larval antigen. (Genta, Neva and Ottesen). Studies aimed at better characterization of this larval antigen that triggers an immediate hypersensitivity reaction are underway.

3. Chagas' disease studies. Data concerning the Chagas' disease patients from the state of Goias who were studied a few years ago has finally been prepared for computer analysis. The HLA-tissue typing has also been completed and has been integrated with the computer analysis of patient data. Various computer printout sheets have been generated and are being checked for accuracy. (Gusmao, Ward of Duke University, Malley and Neva). Based upon this information, some further studies of immunologic response among selected patients of certain HLA-background are planned. (Gusmão, Rezende and Rassi in Brazil, and Neva).

Publications:

1. Raizman, R.E., de Rezende, J.M. and Neva.F.A.: A clinical trial with pre- and post-treatment manometry comparing pneumatic dilation with bouginage for treatment of Chagas' megaesophagus. Am.J. Gastroenterology. 74: 405-409, 1980.

2. Dienstag, J.L., Krotoski, W.A., Howard, W.A., Purcell, R.H., Neva, F.A., Galambos, J.T. and Glew, R.H: Non-A, Non-B hepatitis after experimental transmission of malaria by inoculation of blood. J.Infect.Dis. 143: 200-209, 1981.

3. Berman, J.D. and Neva, F.A.: Effect of temperature on multiplication of leishmania amastigotes within human monocyte-derived macrophages *in vitro*. Am.J.Trop.Med.Hyg. 30: 318-321, 1981.

4. Neva, F.A., Gam, A.A. and Burke, J.: Comparison of Larval Antigens in ELISA test for Human Strongyloidiasis. J.Infect.Dis. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00103-14 LPD

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunological Studies on Toxoplasmosis and Other Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. N. Lunde	Research Zoologist
Other:	E. A. Ottesen	Senior Investigator, LPD, NIAID
	T. E. Nash	Senior Investigator, LPD, NIAID
	T. J. Lawley	Medical Investigator, NCI

COOPERATING UNITS (if any)
Dermatology Branch, National Cancer Institute

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host-Parasite Relations

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.0	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In many parasitic diseases large amounts of parasite antigens and corresponding antibody result in circulating immune complexes. A Clq ELISA has been developed whereby Clq was adsorbed to polystyrene microtiter plates and the complexes bound to Clq from serum were detected by peroxidase labeled anti-human IgG. The sensitivity of the ELISA for detecting immune complexes in sera from filarisis patients was enhanced by the addition of polyethylene glycol to the assay system. antigens and antibody have been labeled with peroxidase for use in detecting specific antigens and antibody in sera from patients with parasitic infections.

PHS-6040
(Rev. 10-76)

Project Description:

Emphasis has been focused on the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of immune complexes. Such an assay can be used to detect immune complexes in sera from patients with parasitic infections without employing radio-labeled reagents. In addition antigens and antibodies are being labeled with peroxidase for use in detecting specific antigens and antibodies.

An ELISA for immune complexes. Clq prepared from normal human serum by the method of Yonemasu and Stroud (1971) was adsorbed to microtiter ELISA plates and horse-radish peroxidase labeled anti-human IgG was used to detect circulating immune complexes bound to the Clq. Sera from patients with autoimmune disorders were obtained from T.J. Lawley (NCI) and sera from patients with filariasis were obtained from E.A. Ottesen (LPD).

Results were compared with the Raji cell assay and ^{125}I-Clq binding assay for immune complexes. These procedures were done in NCI. A technical modification found to be expremely important for detecting immune complexes in sera from patients with filariasis was the addition of polyethylene glycol (PEG) to the sera prior to reacting the bound complexes with the enzyme conjugated antibody. When filariasis sera were tested in parallel with and without the addition of PEG, 10 of 11 sera tested were positive for immune complexes, but only 5 of 11 were positive without PEG.

Antigens and antibodies. The labeling of specific antigens and antibodies will be useful in the identification of either IgG or IgM antibody to these antigens. Toward this end toxoplasma antigen has been labeled with peroxidase and has been used to detect toxoplasma antibody bound to anti-IgM antibody adsorbed to polystyrene plates. *Endamoeba histolytica* antigen has also been labeled and used to detect *E. histolytica* anti-IgG. The successful labeling of these antigens to detect class-specific antibody to the antigens has been a prelude to the eventual labeling of various schistosome antigens and antibody for identification of respective antigen and/or antibody in serum from patients. The main problem encountered in the use of either labeled antigen or antibody has been nonspecific background especially as serum is progressively diluted.

Proposed Course:

It is unclear why the Clq-ELISA for immune complexes in filariasis sera should require PEG when immune complexes can be adequately detected in sera from patients with systemic lupus erythematosus or rheumatoid arthritis without the addition of PEG. This may relate to differences in the nature of the complexes in the two cases. Further studies to define the nature of these complexes are needed to resolve the issue, but operationally the solid phase Clq ELISA with PEG modification can be used for the detection of immune complexes in filariasis sera and should be adaptable for use with sera from other diseases.

ELISA techniques will be used to identify specific antigens and antibodies, especially in schistosomiasis.

Significance to Biomedical Research:

Circulating immune complexes play an important role in the pathogenesis of several human diseases. In parasitic diseases where large amounts of parasite antigen, corresponding antibody and complexes may be present, the study of IC is an important step towards improving serology and in understanding the mechanisms of parasite infection and immunopathology. The identification of these antigens and corresponding specific antibody and how they relate to clinical disease may be helpful in diagnosis, evaluating treatment response and survey serological data. Serological parameters such as presence of antigens and various class-specific antibodies are of help to clinicians in the Clinical Center in determining treatment for the patient presenting a particular clinical situation.

Publications:

1. Wyler, D.J., Blackman, H.J., and Lunde, M.N.: Cellular hypersensitivity to toxoplasmal and retinal antigens in patients with toxoplasmal retinochoroiditis. Am. J. Trop. Med. Hyg. 29: 1181-1186, 1980.

2. Lunde, M.N., Edwards, S.J., Hoffman, G.L., Knight, R.A., Margolis, L., and Powers, K.G. (Translations Committee, A.S.P.): Parasitology translations reported during 1978 and 1979. J. Parasitol. 66: 559-563, 1980.

3. Nash, T.E., Lunde, M.N., and Cheever, A.W.: Analysis and antigenic activity of a carbohydrate fraction derived from adult *Schistosoma mansoni*. J. Immunol. 126: 805-810, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00108-10 LPD

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Biology and Immunogenecity of Malaria Sporozoites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. W. Gwadz	Research Entomologist, LPD, NIAID
Other:	R. S. Nussenzweig	Professor and Head, Division of Parasitology, New York University, School of Medicine
	A. H. Cochrane	Associate Professor, Division of Parasitology, New York University, School of Medicine
	W. A. Krotoski	Chief, Tropical Infectious Disease Research Program, U.S. P.H.S. Hospital, New Orleans

COOPERATING UNITS (if any)

New York University School of Medicine
U.S. Public Health Service Hospital, New Orleans

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12/12	3/12	9/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the immune mechanisms involved in sporozoite-induced infections, and to develop protocols for immunization with sporozoite antigens. Studies underway are concerned with development of methods for immunizing monkeys with Plasmodium knowlesi sporozoites, and characterization of the structure, function and immunogenicity of surface determinants of sporozoites. The biology of the latent exo-erythrocytic stages of the malaria parasite, the hypnozoites, being examined using Plasmodium cynomolgi.

PHS-6040
(Rev. 10-76)

Objectives:

1) To improve methods for immunizing monkeys with sporozoites or sporozoite antigens.

2) To characterize the surface antigens of Plasmodium knowlesi sporozoites responsible for inducing protective immunity in monkeys.

3). To identify and characterize the exo-erythrocytic stages of the malaria parasite responsible for relapse.

Methods:

A) Monoclonal antibodies directed against sporozoites of P. knowlesi have been produced by fusion of spleen cells from sporozoite-immunized mice and NS-1 plasmacytoma cells. Nine hybridoma lines have been isolated and cloned. Antibodies from several of these lines have been purified and Fab fragments prepared.

B) Hypnozoites of the relapsing malaria, P. cynomolgi are being identified and studied in monkey liver tissue using the indirect fluorescent antibody test (IFAT) and in stained specimens.

Results:

A) Of the nine hybridoma lines isolated using the IFAT, five gave positive CSP (circum-sporozoite precipitin) reactions with viable sporozoites of P. knowlesi. In addition, these same five lines reacted with sporozoites of P. cynomolgi in both the CSP and IFA tests.

The sporozoite neutralizing activity of the crude hybridomas, purified antibody and highly purified Fab fragments of the various lines was tested against P. knowlesi. Fab fragments from two lines abolished infectivity of P. knowlesi sporozoites to rhesus monkeys. Patency was delayed after incubation with two others. Fab fragments were found to have higher sporozoite neutralizing activity than either the crude or purified antibodies.

Fab fragments active against sporozoites of P. knowlesi failed to neutralize the infectivity of sporozoites of P. cynomolgi. Antibody from all nine lines has been identified as IgG1.

Metabolic labeling experiments using (^{35}S) methionine indicate that seven of the hybridoma lines recognize antigens with MW's of 42,000 50,000 and 52,000; these same antigens are also recognized by immune serum produced by sporozoite immunization in monkeys. Two hybridoma lines precipitated no specific antigens.

B) Hypnozoites of P. cynomolgi have been identified in liver tissue of rhesus monkeys previously inoculated intravenously with 10^7 sporozoites using a highly specific immunofluorescence technique. Hypnozoites were seen in liver tissue taken from 3 to 105 days after sporozoite inoculation. In one animal, primaquine treatment completely eliminated all hypnozoites.

Proposed Course:

1) Characterization of sporozoite surface antigens will continue, and attempts will be made to isolate and purify the sporozoite antigen responsible for protective immunity.

2) A monkey model using Macaca fascicularis will be evaluated as an alternative to the rhesus monkey system. M. fascicularis monkeys are less expensive than rhesus, are the natural hosts of P. knowlesi, and appear to mount a more uniform immune response. Such a model is necessary for testing purified antigens.

3) Hypnozoite genesis and development will be followed in various strains of P. cynomolgi. In addition, an attempt will be made to demonstrate hypnozoites of P. vivax.

Significance:

A malaria vaccine effective against mosquito-injected sporozoites would be of great importance to human populations at risk in much of the tropical world. Characterization of the functional sporozoite antigens could lead to biological or biochemical synthesis and elimination of unreasonable dependence on mosquito-produced sporozoite antigens for vaccine development.

Publications:

1. Vanderberg, J., and Gwadz, R.: The transmission by mosquitoes of plasmodia in the laboratory. In Kreier, J. (Ed.): Malaria, vol. 2. New York, Academic Press, 1980, pp. 153-234.

2. Krotoski, W.A., Garnham, P.C.C., Bray, R.S., Krotoski, D.M., Killick-Kendrick, R., Draper, C.C., Targett, G.A.T., Gwadz, R.W., Guy, M.W., and Koontz, L. The hypnozoite: New latent stage in primate malaria. Proc. 4th Int. Cong. Trop. Med. Malaria. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00149-06 LPD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Physiology of Anopheles mosquitoes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	F.H. Collins	Staff Fellow, LPD, NIAID
	R.W. Gwadz	Research Entomologist, LPD, NIAID
Other:	L.C. Koontz	Biologist, LPD, NIAID
	P. Graves	Visiting Fellow, LPD, NIAID
	R. Baker	Professor, University of Maryland School of Medicine

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
34/12	25/12	9/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies encompass the physiology, behavior, genetics, and vector competence of mosquito vectors of malaria with particular emphasis on members of the genus Anopheles and factors which mediate the development of the malaria parasite in these insects. a) Techniques have been developed for infecting mosquitoes with in vitro fertilized zygotes of malaria parasites to determine conditions necessary for establishment of the parasite on the mosquito gut. b) Attempts are underway to quantify the effects of malarial infection on the longevity of mosquito vectors. c) Genetic and physiologic factors which determine the susceptibility of mosquitoes to infection are being examined in several important vector species. Chemical mutagens are being used to induce genetic changes which could render a vector species refractory to the malaria parasite and therefore unable to transmit the disease.

PHS-6040
(Rev. 10-76)

Objectives:

A) During sporogony, the protozoan malarial organism changes from a haploid, intraerythrocytic parasite of vertebrates to a diploid, extracellular parasite of mosquitoes. The first events of sporogony occur in the mosquito midgut during the 24 hr following ingestion of a blood meal: gametocyte exsheathment, fertilization, development of the motile zygote (ookinete), and the invasion of the gut epithelium where oocysts develop. The parasite environment during the first 24 hr is the rapidly digesting blood meal. Early sporogony appears to depend on factors in this environment and may determine if a species of Plasmodium will develop in a particular species of mosquito.

B) Malarial infections in vector mosquitoes may affect the daily survival rate and consequently the vectorial capacity of a species. The relationship between the intensity of infection and survivorship is also an important consideration. Initial experiments are designed to determine the effects of infection on vector population life tables.

C) Susceptibility of several species of mosquitoes to malarial infections has been shown to be under genetic control. Similar modes of genetic control of susceptibility have not been demonstrated with anophelines and any of the primate malarias. Selection for refractory and susceptible lines from several important vector species has been initiated; the mode of inheritance will be determined and the physiologic basis for susceptibility will be examined. Chemical mutagens will be used as necessary to induce refractorines and/or phenotypic markers useful for linkage studies.

Procedures and Results:

A) Zygotes of P. gallinaceum have been prepared free of erythrocytes or blood products and fed to mosquitoes at various concentrations and in various media. Zygotes in erythrocyte-free media failed to infect, while increasing concentrations of red cells supported greater levels of infection.

Analysis of erythrocyte constituents indicate that hemin does not support infection while suspensions of globin do support infection.

Zygotes injected directly into the hemocoel of an unfed mosquito form oocysts in the absence of red cells, while similar preparations fed to mosquitoes do not. It appears that the erythrocyte substance is not nutritive, but somehow facilitates invasion of the gut epithelium.

Chickens are most infective to mosquitoes the day before gametocytes peak. This decline in infectivity is due to hemoglobin depletion by the large number of intraerythrocytic parasites.

B) Preliminary studies of the effects of *P. cynomolgi* infections on *An. freeborni* and *An. dirus* indicate a marked increase in the mortality rate during the period when sporozoites rupture from the oocysts and invade the salivary glands. Thereafter, the daily death rate significantly exceeds that of uninfected control groups.

C) Lines of *An. gambiae* refractory and susceptible to *P. cynomolgi* (a parasite similar to *P. vivax*) and *P. coatnegi* (similar to *P. falciparum*) are being selected.

The development of oocysts and sporozoites, and the infections of salivary glands is being studied in *An. dirus* and *An. freeborni* using *P. knowlesi* malaria. Both species support development to the stage where mature sporozoites are present in oocysts. In *An. dirus*, the sporozoites are released and invade the salivary glands. In *An. freeborni*, gland infections seldom occur. The role of the hemolymph and gland receptivity are being examined in the refractory and susceptible species.

Proposed Course:

A) Blood factors which facilitate ookinete invasion of the midgut epithelium will be further analyzed and characterized. The formation of these factors in refractory and susceptible mosquitoes and their role in determining susceptibility will be examined. The chemotactic properties of the midgut epithelium to ookinetes in the presence and absence of erythrocyte factors will be studied *in vitro*.

B) The effects of malarial infections on *An. stephensi* and *An. gambiae* will be examined. In addition, field data on the survival rate of infected mosquitoes from New Guinea and the Sudan will be analyzed and compared with conclusions drawn from laboratory studies.

C) Genetically defined susceptible and refractory lines of *An. gambiae* will be developed and the controlling loci linked to appropriate morphological markers. Similar studies will be initiated with *An. culicifacies*, *An. stephensi*, and *An. dirus*. The feasibility of releasing self-perpetuating refractory lines into field populations will be explored.

Significance to Biomedical Research:

In spite of significant strides in malaria chemotherapy and exciting advances in vaccine research, mosquito control remains the only resonable, effective, economically viable means of malaria control. Mosquito, more specifically *Anopheles* mosquito, behavior, physiology, and genetics are poorly understood. All of these factors influence the competence of mosquitoes as vectors. Disruption of the normal life cycle of the mosquito or alteration of its capacity to transmit malaria could significantly influence the epidemiology of the disease with marked improvement of the health of human populations now at risk.

Publications:

1. Beach, R.F.: Physiological changes governing the onset of sexual receptivity of male mosquitoes. J. Insect. Physiolo. 26: 245-252, 1980.

2. Rosenberg, R.: Ovarian control of blood meal retention in the mosquito *Anopheles freeborni*. J. Insect. Physiol. 26: 477-480, 1980.

3. Rosenberg, R., and Maheswary, N.P.: Forest malaria in Bangladesh. I. Parasitology. Am. J. Trop. Med. Hyg. In press.

4. Rosenberg, R., and Maheswary, N.P.: Forest malaria in Bangladesh. II. Transmission by *An. dirus*. Am. J. Trop. Med. Hyg. In press.

5. Rosenberg, R.: Forest malaria in Bangladesh. III. Breeding habits of *An. dirus*. Am. J. Trop. Med. Hyg. In press.

6. Rosenberg, R., Koontz, L.C., Carter, R.: Infection of *Aedes Aegypti* with zygotes of *P. gallinaceum* fertilized in vitro. J. Parasit. In press.

7. Graves, P.M., and Curtis, C.F.: Susceptibility of *Anopheles gambiae* to *Plasmodium yoelii Nigeriensis* and *Plasmodium falciparum*. An. Trop. Med. Parasitol. In press.

8. Graves, P.M., and Curtis, C.F.: A cage replacement experiment involving introduction of genes for refractorinesss to *Plasmodium yoelii Nigeriensis* into a population of *Anopheles gambiae* (Diptera,Culiadae). J. Med. Ent. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00160-05 LPD

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cell Biology of Entamoeba, Giardia and Other Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. D. Gillin	Senior Staff Fellow, LPD, NIAID
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	T. E. Nash	Medical Officer, LPD, NIAID
	P. D. Smith	I.P.A. Researcher, LPD, NIAID
	F. A. Sher	Head, Cell Biology & Immunology Sect., LPD, NIAID
	M. Suffness	Head, Plant and Animal Products Section, Natural Products Branch, NCI
	D. S. Reiner	Medical Technologist, LPD, NIAID

COOPERATING UNITS (if any) Dr. Andrew Plaut, Gastroenterology Div., Tufts New England Medical Center. Dr. Peter McCann, Head, Cell Biology Div., Merrill Research. John Bognacki, Sloan-Kettering Research Foundation.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Parasite Growth and Differentiation Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
23/12	12/12	11/12

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

I. We have studied possible immune and nonimmune host defense against unrelated human mucosal parasitic protozoa.

A. Giardia lamblia does not activate the alternative complement pathway. With antibody (from goat or rabbit), however, the parasites are killed by complement via the classical pathway. Without complement, such antiserum inhibits parasite attachment.

B. Trichomonas vaginalis activates the alternative complement pathway and is killed by the resultant membrane attack complex.

C. Natural resistance factors: G. lamblia is killed by lactoferrin, an iron-binding protein present in human intestinal fluid and polymorphonuclear cells. Lactoferrin binding to trophozoites was shown by indirect immunofluorescence. Killing activity was thermolabile and the parasites were protected by serum.

II. Drug Studies:

A. Growth of G. lamblia was inhibited by DFMO, an irreversible inhibitor of polyamine biosynthesis. Thus, polyamine biosynthesis appears to be required for growth and may be a chemotherapeutic target.

B. Certain quassinoid protein synthesis inhibitors are potent amebacides.

I. Entamoeba histolylica, Giardia lamblia, and Trichomonas vaginalis, three important human pathogens, are extracellular mucosal dwelling protozoa. Although unrelated, they have biochemical similarities in that all are aerotolerant anaerobes. Earlier, we developed cloning methods for assay of survival of E. histolytica and G. lamblia and a quantitative assay for in vitro attachment of these two parasites. These methods have been utilized extensively in the studies described in the present report.

Trophozoite attachment to host mucosal cells may play a role in disease caused by these parasites. Knowledge we have gained earlier on requirements for attachment has enabled us to assess the possibility that certain host factors may interfere with parasite attachment.

A. Interactions of Giardia lamblia with antibody and complement.

Little is known about innate or immune host factors which may limit infection with these mucosal parasites. E. histolytica trophozoites have been shown to activate the alternative complement pathway (ACP). We have shown that G. lamblia trophozoites are not killed by human or guinea pig complement. Possible explanations are that the trophozoites 1) do not activate the ACP, or 2) they activate the ACP, but are not lysed. However, the parasites are rapidly (< 10 min) killed by complement with goat or rabbit anti-G. lamblia serum, which disproves the second hypothesis. Killing was by the classical complement pathway, as it was ablated by Mg-EGTA which inhibits C1. In the absence of complement, parasites were not killed, but their attachment was strongly inhibited by goat antiserum (50% inhibition at 0.24% serum).

Most patients (81%) with giardiasis have serum IgG antibody to G. lamblia trophozoites. However, this antibody did not appear protective, as sera from patients did not inhibit attachment or kill trophozoites (with complement).

Future Course:

1) To determine whether patient antibody and/or complement facilitates the killing of G. lamblia by white blood cells. 2) To develop an ELISA assay for secretory antibody to G. lamblia and determine its effect on attachment and survival (with P.D. Smith).

B. Activation of the alternative complement pathway by T. vaginalis (with A. Sher).

Trichomonas vaginalis is lysed by fresh, but not heated normal human or animal sera. Our studies demonstrate that T. vaginalis activates the ACP and is lysed by the resultant membrane attack complex. Involvement of the complement system was demonstrated by the failure of human sera deficient in C3 or C8 to mediate parasite killing and by the ablation of lytic activity observed when fresh sera were heated at 56° or treated with EDTA. Fixation of human C3 on the parasite surface was shown by indirect immunofluorescence (IIF). Involvement of the ACP was demonstrated by the inability of properdin-depleted human serum to lyse T. vaginalis and by the normal killing observed with guinea pig serum lacking C4, as well as with normal human or guinea pig serum treated with EDTA and Mg^{2+} to selectively inhibit the classical pathway.

The role of antibody in the ACP has been controversial. Recently, specific natural antibody was shown to play a stimulatory role in activation of this pathway by zymosan. There are many reports of "natural" antibody to _T. vaginalis_. Absorption with _T. vaginalis_ slightly reduces the lytic activity of normal human serum and the addition of heated unabsorbed serum reconstitutes the activity, suggesting that small quantities of "natural antibody" in human sera may stimulate the lysis of the parasite by the ACP. It should be emphasized that antibody is not essential for killing since absorption of guinea pig serum with parasites failed to significantly reduce its lytic activity.

Future course:

Host defences against _T. vaginalis_, a noninvasive mucosal parasite, are not well understood. It is extremely likely that complement is present in menstrual blood where it could act against the parasite. If _T. vaginalis_ is exposed to complement _in vivo_, the question of how the organism survives this natural defense is relevant. One possibility is that the surface of _T. vaginalis_ may undergo an adaptive change _in vivo_ enabling it to avoid lysis by complement. We will attempt 1) to select mutants resistant to complement, and 2) to determine whether parasites can adapt _in vivo_ (in mice) to complement resistance. Preliminary experiments have indicated the feasibility of both of these approaches. Similar experiments will be carried out with _E. histolytica_.

C. Natural defenses against _G. lamblia_.

Innate host mucosal defenses against _G. lamblia_ have not previously been identified. We have found that lactoferrin (Lf), an iron-binding protein in most external secretions (e.g., bile, intestinal fluid, milk), and polymorphs is strongly _Giardia_-cidal. Others have shown that Lf kills susceptible bacteria by binding needed iron. Unlike these bacteria, however, _G. lamblia_ is killed by iron-saturated Lf, implicating a novel killing mechanism. The killing increases with Lf concentration and time, and the activity is very thermolabile. Binding of Lf to trophozoites was shown by IIF. The parasites are protected by serum. Thus, Lf may be a factor in human resistance to giardiasis or in limiting the extent of the disease.

Future Course:

To determine the role of the carbohydrate moieties of Lf in binding to and killing _G. lamblia_ and to elucidate the mechanism of killing (with J. Bognacki).

II. Drug studies: Selected inhibitors may provide new tools for study of parasite biochemistry as well as new chemotherapeutic agents.

A. Inhibition of G. lamblia growth by an inhibitor of polyamine biosynthesis (with P. McCann). Difluoromethyl ornithine (DFMO), a specific irreversible inhibitor of the first enzyme (ornithine decarboxylase, ODC) in polyamine biosynthesis, is highly protective of animals infected with African trypanosomes or with Eimeria. We have found that DFMO also inhibits the in vitro growth of G. lamblia, but not E. histolytica or T. vaginalis. Specificity was shown by restoring growth of G. lamblia with spermidine (a polyamine product of the blocked biochemical pathway). At 2 to 10 mM DFMO, trophozoite growth is inhibited, while at higher concentrations, the parasites are killed. Thus, polyamine biosynthesis appears essential for multiplication of G. lamblia. This may provide a valid biochemical approach for chemotherapy.

Future Course:

To determine levels of polyamines and ODC in susceptible (G. lamblia) and insusceptible (E. histolytica, T. vaginalis) parasites as a function of growth.

B. Killing of E. histolytica by quassinoid protein synthesis inhibitors.

Extracts of Brucea antidysenterica and related plants have been used in Chinese and Ethiopian folk medicine as treatments for dysentery. Quassinoid compounds purified from these plants are protein synthesis inhibitors with antitumor activity. We have found certain of these compounds to have potent amebacidal activity in vitro. One kills E. histolytica at 1/30th the concentration of metronidazole. The relationship between quassinoid structure and amebacidal activity was explored (with M. Suffness).

Future studies:

We hope to determine whether these drugs are effective against hepatic amebiasis in the baby hamster model.

Publications:

1. Gillin, F.D., and Diamond, L.S.: Attachment and short term maintenance of motility and viability of Entamoeba histolytica in a defined medium. J. Protozool. 27: 220-225, 1980.

2. Gillin, F.D., and Diamond, L.S.: Entamoeba histolytica and Giardia lamblia: Growth responses to reducing agents. Exp. Parasitol. 51: 382-391, 1981.

3. Gillin, F.D., and Diamond, L.S.: *Entamoeba hystolytica* and *Giardia lamblia*: Effects of cysteine and oxygen tension upon attachment and survival in culture media. Exp. Parasitol. In press.

4. Smith, P.D., Gillin, F.D., Brown, W.R., and Nash, T.E.: IgG antibody to *Giardia lamblia* detected by Enzyme-Linked Immunosorbent Assay. Gastroenterology 80: 1476-1480, 1981.

5. Gillin, F.D., and Diamond, L.S.: Attachment of *Entamoeba histolytica* to glass in a defined maintenance medium: Specific requirement for cysteine and ascorbic acid. J. Protozool. 27: 474-478, 1980.

6. Gillin, F.D.: The physiology of trophozoite attachment. In Erlandsen, S.L., and Meyer, E.A., (Eds): *Giardia and Giardiasis*: Biology of the organism, pathogenesis of the disease and its epidemiology. Plenum Press. In press.

7. Gillin, F.D., and Diamond, L.S.: Inhibition of clonal growth of *Giardia lamblia* and *Entamoeba histolytica* by metronidazole, quinacrine, and other antimicrobial agents. J. Antimicrob. Chem. In press.

8. Gillin, F.D., and Sher, A.: Activation of the alternative complement pathway by *Trichomonas vaginalis*. Inf. Immun. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00161-04 LPD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immunochemistry of Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Others:	
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A.W. Cheever	Assistant Chief, LPD, NIAID
E.A. Ottesen	Senior Investigator, LPD, NIAID
Nasis-ud-Din	Associate in Biological Chemistry, Laboraory of Carbohydrate Chemistry, Harvard Medical School
F.D. Gillin	Senior Staff Fellow, LPD, NIAID
L.S. Diamond	Head, Parasitic Growth & Differentiation, LPD, NIAID
M.N. Lunde	Research Zoologist, LPD, NIAID
R. Hussain	Guest Worker, LPD, NIAID
D.B. Keister	Biologist / N. Anwar Visiting Fellow, LPD, NIAID

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SECTION
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TOTAL MANYEARS: 26/24	PROFESSIONAL: 14/12	OTHER: 22/24

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The identification and clinical utilization of parasite derived antigens remains a major goal of the laboratory. PSAP, a glycoprotein obtained from adult S. mansoni was purifed by means of coloumn and lectin chromatography. Labelled PSAP was utilized to measure IgM and IgG responses.In defined groups of patients acute infection could be differentiated from chronic infection by the predominate IgM responses when compared to total antibody responses. Giardia lamblia following labelling with either Iodogen or lactoperoxidase secrete about 70% of the label into the culture media after about 24 hours. Identification of the secreted products is continuing. Excretory-secretory products of B. malayi were used to define antibody responses in infected patients.

Project Description:

The identification and utilization of specific parasite antigens remains a primary concern of this laboratory. Two major specific S. mansoni carbohydrate antigens have been identified. PSAP was purified from a trichlorocetic acid soluble fraction of adult S. mansoni by DEAE chromatography. Studies utilizing this fraction in ELISA tests and later radioimmunoassays revealed that antibody responses in humans were correlated with the egg excretion rates in patients not acutely infected. PSAP was radiolabelled with Bolton-Hunter reagent and found to be uncontaminated in initial experiments. On acrylamide it migrated as a 130,000-90,000 polydisperse PAS positive material. Later experiments revealed non-immunogenic contaminants that were removed by either sepharose 4B chromatography in urea containing buffers or lectin chromatography. PSAP bound to Con A, lentil lectin, R. communis and wheat germ agglutinin indicating the presence of mannose or glucose, galactose and N-acetylglucosamine. Using Con A chromatography the non-adhering fraction contained the contaminants. When other preparations of PSAP were used, purification was acheived before labelling using sepharose 4B chromatography and monitoring by radioimmunoassay. After labelling PSAP was an uncontaminated poly disperse antigenic material as judged by gradient accrylamide electrophoresis and coloum chromatography. Using radiolabelled antigen in radioimmunoassays, PSAP was found to be present in S. mansoni eggs , and to a lesser extent in cerceriae and schistosomules. Additionally, both male and female worms released PSAP into culture media and antibody responses were present in unisexually and bisexually infected animals. Responses were greater in bisexually infected animals.

PSAP levels in man were studied in defined groups of infected patients using two radioimmunoassays. Labelled PSAP after combining with antibody was precipitated with polyethylene glycoland (PEG) staph A. Using a double antibody precipitation test and mercaptoethanol destruction of IgM antibodies PEG was found to precipitate both IgG and IgM antibodies but staph A combined with IgG. Since antibody responses were previously found to be dependent on egg excretion, the ratio of staph A/PEG was determined for each patient at a dilution where antibody was not in excess. Acutely infected patients had predominantly IgM responses and therefore low ratios whereas chronically exposed patients had higher levels of IgG and higher ratios. This easily distinguishes between chronic and acutely infected patients.

The other carbohydrate antigen, GASP, is a large molecular weight proteoglycan present in the intestinal cells of the schistosome and regurgitated into the blood stream. Previous studies defined humoral responses to GASP in humans. However, the ultimate fate of GASP and the factors modulating degration and removal have not been studied. The clearence of GASP injected intravenously into infected and non-infected mice was dependent on the amount of antigenic binding activity at the time of injection. Binding activity was in turn related to both the duration of infection and the worm burden. Binding levels (presumably antibody & immune complexes) were increased 2 weeks after infection and this was associated with an accelerated clearence rate. Heavily infected animals had less antigen binding and slower clearence rate compared to lightly infected animals. Both infected and non-infected mice cleared the antigen in the liver. Most of the label then appeared in the urine as a small m.w. non-antigenic material.

Studies defining excretory-secretory (E-S) antigen of B. malayi were completed. (Anwar, Hussain, Nash, Ottesen). Labelled antigens were employed to determine antibody response in human infection.

Studies were initiated using surface labelled G. lamblia. Parasites could be labelled and maintained alive. About 70% of the label was shed into the media over the next 18 hours. Specific membrane and secreted materials have been identified. Cytotoxicity studies of human peripheral blood leukocytes for G. lamblia continues (Smith, Nash, Gillen).

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1. CHeever, A.W., Duvall, R.H., Minker, R.G. and Nash, T.E.: Hepatic fibrosis in rabbits infected with Japanese and Phillipine strains of Schistosoma japonicum. Am. J. Trop. Med. Hyg. 29:1327- 1339, 1980.

2. Nash, T.E., Lunde, M.N., and Cheever, A.W.: Analysis and antigenic activity of a carbohydrate fraction derived from adult schistosoma mansoni. J. Immunol. 126:805-810, 1981.

3. Smith, P.D., Gillin, F.D., Brown, W.R., and Nash, T.E.: IgG antibody to Giardia lamblia detected by enzyme-linked immunosorbent assay. Gastroenterology. 80:1476-1480, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00162-05 LPD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Cytology of Host-Parasite Interactions in Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Aspects of the cell biology and immunology of host-parasite interactions among several intra- and extracellular parasitic protozoa are being investigated. Leishmania sp. and Trypanosoma sp. are being used as models of intracellular and extracellular parasitism, respectively. Emphasis is placed upon 1) determining some of the basic chemical and antigenic properties of parasite surface membranes; 2) ascertaining the nature and extent of the interactions of parasite surfaces with specific host cell types; 3) defining the basic mechanisms involved in the intracellular survival and multiplication of parasites within host cells; and 4) attempts to determine the means by which parasites circumvent host immuno-defense systems. Techniques employed in these studies include; subcellular fractionation, ultracyto-and immunochemistry, electron microscopy, chromotography, polycrylamide gel electrophoresis, lectin assays, radio isotope labeling , gel immunoassays, and in vitro cell culture.

Project Description and Objectives:

How parasites evade host immune responses, are nourished, multiply, and eventually destroy the host are questions being asked in this investigation. In as much as all initial interactions between host and parasite occur at the level of their respective cell membranes, and understanding of the nature, and physiology of these membranes is essential. It is to this end that parasite chemical composition, antigenic nature and physiology are being investigated specifically with regard to leishmaniasis and trypanosomiasis. Further, parasite interaction with specific host cell types and immune systems are being studied to ascertain the role of parasite surface membranes in infectious disease processes.

Methods Employed:

Techniques used include: 1) fine structure (transmission and scanning electron microscopy, ultrastrucural cyto-and immunochemistry, freeze fracture and etching methods, and autoradiography); 2) subcellular fractionation; 3) analytical and preparative polyacrylamide slab gel electrophoresis and isoelectric focusing; 4) antibody and lectin binding assays; 5) radio isotope labeling and autoradiography; 6) chromatography (gel permeation, affinity, thin layer, and gas); 7) qualitative and quantitative gel and radio immunoprecipitin assays; and 8) *in vitro* cell cultures.

Isolation and characterization of subcellular components from *Leishmania* and *Trypanosoma*:

1. Antigenic properties of surface membranes isolated from *Leishmania donovani* (Dwyer).

As demonstrated previously in this laboratory, Triton X-100 extracts of isolated *Leishmania donovani* pellicular membranes (PM-TX) contain ~ 40 peptide/glycopeptide constituents ranging in molecular weight from $\leq 1.5 \times 10^4$ to $> 10^5$ daltons. Antisera were raised in rabbits against both whole isolated PM and several detergent soluble PM constituents. These rabbit anti-PM sera were reacted against PM-TX constituents in gel diffusion, immunoelectrophoresis (IEP) and crossed-immunoelectrophoresis (CIEP) assays. Cumulative results of the IEP and CIEP analyses demonstrated that the PM-TX contained a minumum of 32 antigenic constituents. Of these, most possessed a slightly anodic charge at pH 8.6. No cathodic migrating antigens were observed in these gels. These results demonstrate the antigenic complexity of the *L. donovani* PM. In gel diffusion reactions sera from more than 4 dozen acute cases of Kala azar from widely divergent geographic areas (i.e. Greece, Turkey, Sudan, Kenya, and India) were tested for their preciptin reactivity against PM-TX. All of these sera gave a positive specific precipitin reaction with PM-TX, and all gave precipitins of apparent identity against the same antigen(s). Further, these patients sera all gave precipitins of identity with 12 separate pools of *L. donovani* acutely infected hamster sera in reactions against PM-TX. These results indicate that both acutely infected patients and experimental animals recognize and mount a specific precipitating antibody response to a parasite surface membrane antigen(s). Further, these results also suggest that this PM antigen(s) is a common constituent of both promastigote and amastigote developmental stages. Rabbit antisera raised against this antigen gives a reaction of identity with both Kala azar and infected hamster sera. Results to date indicate that this gel immunoprecipitable antigen is an externally disposed PM glycoprotein of apparent molecular weight $\cong 8 \times 10^4$ daltons. Immunoprecipitin results similar to those described above for Kala azar and hamster sera have also been obtained from reactions between PM-TX and sera from patients with diffuse cutaneous leishmaniasis (i.e. from the Dominican Republic, Leishmania sp.?). The latter results suggest that this PM component might represent a common externally oriented surface constituent of all leishmanial species (i.e. a generic antigen) and thus be responsible at least in part, for their serologic cross reactivity.

2. Identification of the externally oriented surface membrane antigens of Leishmania donovani promastigotes and amastigotes (Dwyer).

Externally disposed surface membrane proteins/glycoproteins of L. donovani promastigotes and amastigotes were radiolabeled via the ^{125}I-Iodogen method. Quantitative results obtained in these studies demonstrated that > 99% of all covalently bound ^{125}I activity was associated with the parasite surface membrane. Autoradiographic data obtained from SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) separated samples indicated that > 22 PM constituents ranging in apparent MW from $\leq 6.5 \times 10^3$ to $> 2.8 \times 10^5$ daltons were specifically labeled via this technique. Membrane fractions of the labeled cells were extracted with Triton X-100, and these extracts were used in radio-immunopreciptation and binding assays. Immune complexes formed in vitro between the detergent solubilized labeled membrane constituents and various rabbit, human and hamster sera were bound to and precipitated by fomalin fixed Staph. A cells. Such precipitated complexes were detergent solubilized, separated via SDS-PAGE, and detected by autoradiography of the dried electropherograms. Multivalent anti-promastigote PM sera precipitated a total of > 17 promastigote PM antigens ranging in ~ MW from $< 11.5 \times 10^3$ to $\bar{1}.6 \times 10^5$ daltons. Of these, 8 were much more strongly labeled and had ~ MW of 2.1, 2.6, 3.5, 3.6, 4.4, 5.8, 7.0, and 10×10^4 daltons. Similar, albeit quantitatively different, results were also obtained with ^{125}I-labeled amastigotes and the anti-promastigote PM sera. These results indicate that both parasite developmental stages share a minimun of 17 externally disposed surface membrane antigens. Conclusions drawn from the later experiments were verifed by results obtained fron both homolgous and reciprocal radio-precipitations using ^{125}I- labeled pro-and amastigotes and rabbit anti-whole promastigote and anti-whole amastigote sera.

Eight major bands were detected in autoradiograms of reactions between ^{125}I-labeled promastigotes and sera from acute Kala azar patients (KAS). Identical results were obtained from reactions between KAS and ^{125}I-labeled amastigotes. These results further demonstrate that both parasite developmental stages share some common surface membrane antigens. Results identical to those above were also obtained with the 2 radio labeled parasite stages and sera from L. donovani terminally infected hamsters. These results indicate that both infected patients and experimental animals mount similar circulating IgG immune responses to the same externally disposed parasite surface membrane antigens. To one of these antigens, an 8 x 10^4 dalton glycoprotein, both the human and the hamster make a precipitating IgG response (see gel immunoprecipitin results Section 1 above). The remaining 7 membrane antigens (~MW $\leq$ 13,000 to $\geq$ 110,000 daltons) might be present as partially saturated circulating antigen- antibody complexes in sera from both infected hosts. Alternatively, these antigens might stimulate the production of a nonprecipitating sub-class of IgG in the infected host. The latter hypotheses remain to be tested experimentally.

3. Iodination of the external surface membrane components of Leishmania tropica (Gardiner and Dwyer).

Externally disposed surface membrane proteins and glycoproteins of Leishmania tropica promastigotes were labeled with ^{125}I via both the lacto-peroxidase - glucose oxidase method and by the use of the insoluble catalyst Iodogen. Labeled cells were solubilized and separated via SDS-PAGE. Autoradiograms of such gels revealed a minumum of 19 labeled bands ranging in ~MW from 10^4 to $\geq$ 1.1 x 10^5 daltons in Iodogen catalyzed samples and a minumum of 21 bands (~MW $\leq$ 10^4 to $\geq$ 2.6 x 10^5 daltons) in samples iodinated via lactoperoxidase. Less than 2% nonspecific (adsorption) labeling of cells was observed in both of these methods. Further, $\geq$ 95% of all activity was present in the 100,000g pelletable fraction of labeled and disrupted cells demonstrating minimal radio incorporation by cytoplasmic proteins. Protease treatment of labeled cells caused both the release of bound radioactive material into the suspending medium and the diminution and degradation of the resultant autoradiographic patterns of digested cells. Trypsin reduced the amount of cell bound ^{125}I activity by ~30%, chymotrypsin and pronase by ~45% thus further demonstrating that externally oriented cellular constituents were labeled via the procedures employed.

4. Characterization of common surface membrane antigens of Leishmania (Gardiner and Dwyer).

Using the Iodogen method for surface iodination of promastigotes, various strains of L. tropica and other Leishmania species (e.g. L.mexicana, L. baziliensis, L. panamaensis, etc.) are being compared for similarities in their iodinable surface constituents. In vitro immunoprecipitation of Triton extracts of ^{125}I labeled promastigotes with antisera against isolated L. donovani surface membranes indicates that all Leishmania species studied to date share one or a number of cross-reacting surface antigens falling in the range of ~MW from 2.2 to 6.5 x 10^3 daltons. Such cross-reactivity suggests that highly specific antisera to certain surface components of one Leishmania species will prove useful in the cell biology analysis of the human pathogenic species of Leishmania. To this end, methods for elucidating antibody responses to leishmanial antigens in Balb/C mice, and an ELISA method for detection of these responses, are being perfected with a view of generating monoclonal antibodies to leishmanial surface antigens.

5. Shed membrane antigens from Leishmania promastigote cultures (Kaneshiro, Gottlieb, & Dwyer).

Antisera to purified L. donovani surface membranes precipitate 2 major carbohydrate containing antigens (SMA-A and SMA-B) found in cell-free microfiltrates from promastigote cultures, but not in fresh media. These major antigens have been identified in several serum-supplemented tissue culture media as well as a chemically-defined leishmanial medium with bovine serum albumin (BSA), RE-III, and in the latter medium lacking BSA. Production of these SMA were characterized with culture age and found to be greatest in serum containing media >>in RE-III > RE-III without BSA. Immunoelectrophoretic (IEP) analyses demonstrate that SMA-A and -B form contiguous precipitin lines indicating common antigenic sites. The faster migrating component, SMA-A , was separated from SMA-B by membrane filtration; SMA-A was retained by Amicon PM-10 and excluded by XM-50. SMA-B was purified from residual SMA-A by Ricinus-120 lectin affinity chromatography. Anti-membrane IgG preabsorbed with SMA-A failed to precipitate SMA-B thus confirming the presence of common antigenic sites. Further, Triton extracts of surface membrane preparations analyzed by IEP or crossed - IEP against homologous antiserum preabsorbed with SMA-A lose the major immunopreciptin arcs or peaks seen with unabsorbed serum. These results indicate that SMA-A and -B and at least two major membrane antigens are related.

Phenol-water or water-butanol extracts of intact L. donovani promastigotes contain materials which give similar IEP patterns to those of spent growth media described above. SDS-PAGE electropherograms of this extract demonstrate a low molecular weight component which stains for lipids, carbohydrates, and peptides. In the latter regard, this component is similar to surface membrane associated lipopeptidophosphoglycans (LPPG) of Trypanosoma cruzi and Acanthamoeba. In both of the latter, the lipid moiety contains inositol and ceramide. In that regard, we have identified and partially characterized an inositol phosphosphingolipid which constitutes ~15% of the L. donovani promastigotes total phospholipids. Extracts from ^{14}C-inositol grown cells were analyzed via IEP and autoradiography. Results have indicated that a slower migrating component that corresponds to SMA-B above was metabolically labeled with ^{14}C-inositol. It appears that the 2 major antigens present in spent culture media are of cell surface origin and although antigenically cross-reactive they are chemically distinct. Our results suggest that the shed antigens might represent an LPPG in the cells surface and the LPPG may contain this unique inositol sphingolipid.

6. Cellular lipid composition of Leishmania promastigotes (Wassef & Fioretti).

Lipids are an integral part of the membrane structure of cells. In that regard the total lipid composition of Leishmania are being investigated. Results to date indicate that extractable lipids of L. donovani constitute ~32.3% of the total cellular mass. Of these, neutral lipids compose 24.1%, glycolipids ≅ 7.2%, and the remainder (68.7%) are phospholipids. The composition of the phospholipid fraction varied significantly among various subcellular fractions. Purified surface membranes contained phosphatidylethanolamine (PE) as a major phospholipid which accounted for ~33.2% of the total fraction while phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and cardiolipin (DPG) comprised 13.3, 18.5, 10.4, and 1.0%, respectively. A major unidentified phospholipid accounted for the remaining 24% of this fraction. In contrast, a kinetoplast/mitochondrion fraction contained PC as a major phospolipid as well as significant quantities of DPG and PG. PE was a minor component of this fraction. The unidentified phospholipid was also present in this fraction. A granule/vesticle fraction, on the other hand contained a phospholipid profile similar to that of whole cells above. Attempts to characterize the unknown phospohlipid are underway, and preliminary results indicate the presence of inositol, phosphorous, and sphingolipid moieties in addition to at least one carbohydrate ligand. Quantitative characterization of all structural lipid components will facilitate the understanding of membrane structure - function relationships concerning the physiologic properties of L. donovani.

7. Lipolytic enzymes of L. donovani surface membranes (Wassef & Fioretti).

Phospholipases have long been suspected as having a role in the pathology of trypanosomatid infections. In this regard, and in relation to further biochemical characterization, the presence of various lipolytic enzymes was assessed in surface membranes isolated from L. donovani promastigotes. To date, the activity of at least 3 distinct lipolytic enzymes have been demonstrated in these membranes. One, a phospholipase C-like activity hydrolyzed PE into diglyceride and ethanolamine phosphate. In the presence of 2mMCa++, this enzyme displayed optimal activities of 3.3 and 2.8 micro-mole/mg protein/min at pH 7.0 and 8.5, respectively. This enzyme was thermo-labile and was inhibited by Na-deoxycholate. Phospholipase-C activities have been implicated in other systems as playing a key role in exocytosis.

A second enzyme, a phospholipase A_2 was also present in the membranes at high activity. This membrane bound A_2 activity hydrolyzed PE into free fatty acid and lyso-PE. Such activity is important in cellular deacylation-reacylation cycles, in the remodeling of fatty acid composition esterified to membrane phospholipids, and in the fluidity of cell mambranes. The leishmanial enzyme required 2mMCa++ and 1.25mM Na-deoxycholate for maximum activity. This enzyme was heat stable, and had optimum specific activities of 3.8 and 4.3 micro-moles/mg protein/min at pH 9.5 and 7.0 respectively.

Further, a phospholipase A_1 activity was also demonstrated in the isolated membranes. This enzyme displayed maximum specific activities at pH 5.0 and 7.0 (2.3 and 3.0 micro-moles/mg protein/min, respectively).

Characterization of these lipolytic enzymes should further implement our understanding of the physiology and the structure - functional relationship of the L. donovani surface membrane.

8. Freeze - fracture and - etching of L. donovani surface membranes (Pinto DaSilva & Dwyer).

Fine structure freeze - fracture and - etching studies of intact and isolated L. donovani surface membranes have continued to further discern the supramolecular structure of these membranes. The relationship of intramembranous particles (IMP) to subtending inner lamina attacted microtubles was established. Currently, studies are in progress using various lectins and antibodies to ascertain what role, if any, cross-linking externally disposed constituents has on the molecular (i.e. IMP) reorganization withnin the plane of the membrane lipid bilayer.

9. Localization and characterization of *L. donovani* pellicular membrane phosphate monoester enzymes (Gottlieb & Dwyer).

Studies have continued concerning the enzymes characteristic of the *L. donovani* surface membrane. To date, all of the enzymes elucidated in these studies have been phosphate monoesterases (i.e. acid phosphatase, a 5' and a 3'- nucleotidase, ATPase, and a adenylate cyclase). During the course of further investigation concerning the externally oriented surface membrane bound acid phasphatase, we observed that acid phosphatase was also readily detectable, extracellularly, in the growth medium of promastigotes. The extracellular enzyme was soluble, actively released or secreted by log phase cultures, and not attributable to cellular lysis. It accounts for $\geq$ 90% of the total enzmic activity present in log phase cells. Preliminary estimates indicate that the soluble enzyme has as apparent MW of $>3 \times 10^5$ to $\geq 10^6$ daltons using membrane ultrafiltration and Sephacryl S-300 exclusion chromatography, respectively. As with the membrane bound enzyme, the extracellular acid phosphatase had a pH optimum of 4.5, had no activity in the alkaline range, and was completely inhibited by 0.5mM NaF. However, the 2 enzymes could be distinguished on the basis of substrate specificity. The extracellular enzyme having a much broader range of substrate specificities as well as having significantly higher relative activities with substrates commonly recognized by the membrane bound enzyme. Studies are in progress to further purify, characterize, and ascertian the physiologic function of this unique extracellular enzyme.

10. Cell surface antigen "capping" in African bloodstream trypanosomes (Gardiner & Finerty).

Living bloodstream forms of a clone of *Trypanosoma rhodesiense*, raised in Type II nude mice, demonstrated redistribution/capping of surface bound antigens subsequent to treatment with rabbit anti-clone specific antiserum and fluorescein labeled anti-rabbit immunoglobins. In parallel studies using ferritin-labeled conjugates at the fine structure level however, large scale removal of the trypanosome surface coat was not observed but some accretion of label was noted around the flagellar reservoir as well as the production of labeled plasmanemes. These contradictory results do not appear to be due to bound host immunoglobin or complement component C_3 or the production of Forsman - like antibodies by inoculated rabbits.

Antibodies to separated surface coat material from the same trypanosome clone are being raised to reexamine this question and shed light on the arrangement and potential mobility of surface coat proteins in *T. rhodesiense*.

11. Isolation and characterization of *L. donovani* Kinetoplast - mitochondria (Weinbach & Dwyer).

Studies concerning the terminal respiratory metabolism and constituent enzymes (i.e. ATPase, succinic dehydrogenase, etc.) of isolated *L. donovani* kinetoplast - mitochondrion fractions have continued. For details see Dr. Weinbach's report (i.e. Z01 AI 0098-25 LPD).

Proposed Course:

1. Various specific antibody, lectin, and radiochemical probes will be used furhter to qualitatively and quantitatively elucidate the nature of the intact *Leishmania sp.* and *Trypanosoma sp.* surface membranes.

2. Specific antibody and lectin affinity chromatography and radiolabeling techniques will be used to isolate specific parasite PM constitutents for their subsequent detailed chemical and antigenic analyses.

3. Specific antibodies against *Leishmania* PM antigens will be used as probes to ascertain the presence and identity of parasite antigens on the surface of infected macrophages *in vitro*. Such antibodies might also be tested for their efficacy to suppress parasite growth and multiplication in cultured macrophages *in vitro*.

4. Lipid compositional analyses of the isolated *Leishmania donovani* PM have been initiated and these will be pursued. The enzymology of lipid biosynthesis and degradation in such membranes will also contiune specifically with regard to characterization of membrane bound lipases.

5. Studies will continue concerning the nature and cellular origin of parasite exoantigens present in *in vitro* cultures and their possible role in the state of host immunoresponsiveness.

6. Further characterization of membrane bound phosphate monoesterases will also be continued.

Significance:

Basic research being pursued in this project concerning the molecular and structural composition of specific parasite surface membranes and their interactions with specific host cell types might provide a basis for future immunoprophylaxis programs and regimens and, or, a more rational approach toward the development of effective delivery systems for chemotherapeutic treatment of these diseases of man.

Publications 1980 - 1981

Papers:

1. Dwyer, D.M. : Isolation and partial characterization of surface membranes from *Leishmania donovani* promastigotes. *J.* Protozool. *27*, 176-182 (1980).

2. Dwyer, D.M. and D'Alesandro, P.A.: Isolation and Characterization of pellicular mambranes from *Trypanosoma lewisi* bloodstream forms. *J. Parasitol*. *66*, 377-389 (1980).

3. Dwyer, D.M. : Structural, chemical, and antigenic properties of surface membranes isolated from *Leishmania donovani*. *In* Slutzky, G.M. (Ed.): *The Biochemistry of Parasites*. Pergamon Press, New York. pp. 10-28 (1981).

4. Breman, J.D. and Dwyer, D.M. : Expression of *Leishmania* antigen on the surface membrane of infected haman macrophages *in vitro*. *Clin. Exp. Immunol.* *44*, 342-348 (1981).

5. Berman, J.D., Fioretti, T.B., and Dwyer, D.M.: *In vivo* and *in vitro* localization of *Leishmania* within macrophage phagolysosomes: use of colloidal gold as a lysosomal label. *J. Protozool*. *28*. In Press.

6. Gottlieb, M. and Dwyer, D.M. : Phosphomonoesterase activities at the surface mambrane of *Leishmania donovani* promastigotes. *In* Slutzky, G.M. (Ed.): *The Biochemistry of Parasites*. Pergamon Press, New York. pp 30-45 (1981).

7. Gottlieb, M and Dwyer, D.M. : Protozoan parasite of humans: surface membrane with externally disposed acid phosphatase. *Science* *212*, 939-941 (1981).

8. Gottlieb, M. and Dwyer, D.M. : *Leishmania donovani*: identification localization, and partial characterization of acid phosphatase activity on the surface membrane of promastigotes. *Exp. Parasitol*. In Press.

9. Dwyer, D.M. : Acceptance of the Henry Baldwin Ward Medal. *J. Parasitol.* *67*. In Press.

10. Gottlieb, M. and Dwyer, D.M. : Identification and partial characterization of an extracellular acid phosphatase activity in cultures of *Leishmania donovani* promastigotes. *Molec. Cell. Biol*. Submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00174-04 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Culture, physiology and antigenic analysis of sexual and asexual erythrocytic malaria parasites

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Animals have been sucessfully vaccinated against sexual and asexual malaria parasites. In addition, Plasmodium falciparum, the major malaria of man, has been grown in culture for the production of asexual stages and gametocytes.The present study will 1) Improve culture conditions for the production of large numbers of gametocytes, gametes and merozoites of P. falciparum; 2) Study of the physiology of exflagellation, fertilization and invasion of red cells by malaria merzoites; 3) Characterization structure, and function and immunogenicity of surface determinants on gametes and other mosquito midget stages and merozoites; 4) Evaluate gamete vaccines in model systems for identification of the best antigens and adjuvants. 5) Characterize red cell membrane of infected red cells.

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Objectives

I. Gametocytes and Gametes

A. P. gallinaceum/chicken

1. Identify unique and common surface proteins on male and female gametes. Determine the effect of monoclonal antibodies (including Fab' fragments) on fertilization. Determine if isolated surface proteins will induce transmission-blocking immunity in chickens.

2. Study immunogenicity of stages of sporogonic cycle (gametes, zygotes and ookinetes). Determine unique immune mechanisms that could block sporogony during development of zygotes or ookinetes. Identify unique surface proteins synthesized during these stages.

B. P. knowlesi/rhesus monkey

1. Determine immune mechanisms and course of immunity after vacination including boosting of immunity.

2. Identify surface antigens on the mosquito midgut stages.

3. Identify common antigens between gametes and merzoites.

C. P. falciparum in culture

1. Study gametocytogenesis in culture including effect of cAMP and time in culture on different isolates and clones of isolates.

2. Improve methods for mosquito infectivity.

3. Identify monoclonal antibodies against surface determinants on gametes that will block mosquito infectivity.

4. Analyse the developmental origin, stage specificity, and cellular location of antigens of the sexual stages.

5. Analyse the stage specificity of antigens recognized by sera from individuals exposed to endemic P. falciparum.

II. Asexual Parasites

A. Merozoites and red cell invasion

1. Study red cell determinants for invasion and effect of surface modification (enzyme and antibody) on invasion. Identify merzoite receptors for red cells by use of Fab' fragments of monoclonal antibodies. Identify contents of apical organelles involved in invasion. Produce mutants of P. falciparum with different host cell specificity (e.g. invade rhesus red cells).

B. Asexual erythrocytic parasite

1. Identify components on the surface of P. knowlesi and P. falciparum infected red cells and changes in infected membrane.

2. Study mechanism of sequestration by P. falciparum infected red cells. Determine red cell membrane and endothelial components involved in sequestration. Determine effect of immune sers on sequestration. Determine the effect of immunity to knowlesi on parasite survival in vivo in the P. coatneyi and P. fragile / rhesus models.

Studies on asexual and sexual parasites are interrelated: Structure and function of parasite determinants will be studied in the framework of parasite physiology. Characteristics of clonal parasites in culture will be used for studies of sexual and asexual parasites. Common antigens may exist between sexual and asexual parasites.

Methods

1. Continuous culture of red cell stages of *Plasmodium falciparum*; isolation of sexual and asexual stages; purification of merzoites and gametes.

2. Use of model systems: *P. gallinaceum*-chicken for study of gametes and and gamete immunity; *P. knowlesi*-rhesus for study of mechanism of invasion, antigenic variation and gamete immunity; *P. coatneyi*-rhesus for study of immunity against a sequestered parasite.
3. Use of polyspecific antibody to parasite antigens for immune precipitation; high-resolution gel electrophoresis and electrofocussing separation techniques; ^{125}I lactoperoxidase surface labelling; labelling of sugars and other components by various methods; general metabolic labelling of all parasite components; and lectin column purification of malarial antigens.
4. Monospecific antibody produced in hybridomas for analysis of parasite surfaces and isolation of surface determinants.
5. *P. falciparum* parasitized red cell with (K+) and without (K-) knobs will be layered over human endothelial cells in culture and the percent adherence measured.
6. Use of membrane feeding to feed malaria infected blood to mosquitoes in the presence of substances, e.g. hybridoma antibodies, to be tested for their effect on infectivity of the parasites to mosquitoes.
7. Subcellular fractionation of parasites.

Results of Research

I. Sexual Stages

1) Studies on gametocytogenesis by *P. falciparum in vitro* show large differences in the spontaneous rate of formation of gametocytes by different isolates. Those isolated from South America and South Asia tend to produce the highest numbers compared to isolates from Africa. However isolates are not stable in their rate of gametocyte production, some increasing and others decreasing with length of time in continuous culture.

2) Using preparations of *P. gallinaceum* zygotes fertilized *in vitro* have we have shown that these are infective to *Aedes aegypti* mosquitoes. The infectivity of the zygote may be greatly reduced or eliminated by sera from chickens immunized against sexual stages of the parasite. Antibodies which block fertilization are not dependent on complement for their effect; the antizygote antisera on the other hand depend on a 56°C inactivated component for most of their effect.

Antisera may also block the infectivity of ookinetes to mosquitoes. This effect is largely retained in sera inactivated at 56°C.

3) *P. gallinaceum* zygotes are equally infectious when fed to mosquitoes in the presence of heat inactivated and non-inactivated sera from non-immune chickens, ducks or turkeys. Non-inactivated mammalian sera block zygote infec-

tivity. Ookinetes are susceptibile to heat labile components in both avian and mammalian sera. It is postulated that zygotes are protected from the alternate pathway of complement activation in avian sera. Ookinetes are susceptible.

4) Immunochemical characterization of midget stages of P. gallinaceum using stage specific antisera against highly purified midgut stages show microgametes are identical by criteria of immune competition and by electrophoretic mobility.

i) 6 major surface components on zygotes are readily labelled with and precipitated by immune sera. Those components with M.W. greater than 60,000 are also found on the surface of male and female gametes. Those surface labelled components of less than 60,000 M.W. are shared by zygotes and female gametes but are absent from the male gametes.

ii) One of the surface of labelled components (M.W. 185,000) on the zygote is found only when the parasites have been exposed to serum and has been shown to be derived from the serum. All other bands are of parasite origin.

iii) During transformation of zygotes to ookinetes in vitro all but one of the surface labelled bands are lost . A single new surface component of about 30,000 M.W. appears on the ookinete.

iv) Gametocytes metabolically labelled with ^{35}S methionine during transformation into gametes show de novo synthesis of most components precipitated by immune sera from the gametes. Synthesis of these components could not be detected in gametocytes or asexual parasites. Among the components synthesized de novo by the gametes is one precipitated by the hybridoma antibody 11C7 and shown previously to participate in antigamete immunity.

v) The profile of immunoprecipitable components metabolically labelled with ^{35}S methionine underwent a total change during the transformation of zygotes to ookinetes. Among those which were not synthesized 8 hours after fertilization was the component precipitated by 11C7. This component thus appears to be synthesized only by gametocytes or zygotes.

5) Using the monoclonal antibody to gametes of P. gallinaceum a component has been immunoprecipitated from zygotes separated by SDS polyacrylamide gel electrophoresis and used to immunize chickens. Preliminary results indicate that one immunized bird has produced antigamete antibodies capable of mediating partial suppression of infectivity to mosquitoes.

6) Hybridomas have been developed against gametocytes of P. falciparum and identified by immunofluorescent test. These await further characterization.

7) Transmission blocking immunity persists in rhesus monkeys immunized over 4 years ago with a mixture of P. knowlesi gametes in FCA. Both intact and splenectomized monkeys continually fail to infect mosquitoes after challenge infections.

Repeated infections of non-immunized monkeys do not produce detectable antibody levels capable of interrupting transmission. However similar repeated infections in immunized animals appear to boost antibody levels and insure complete immunity.

Monkey immunized with sporozoites, trophozoites or merozoites and exhibiting protection aganist those stages do not show transmission blocking immunity.

I. Asexual Parasites

Erythrocytes infected with the late stages of the human malarial parasite, *P. falciparum*, attached to human endothelial cells via knob-like protrusions on the surface of infected erythrocytes. Not all endothelial cells bound infected erythrocytes. Infected erythrocytes did not bind to fibroblasts. The binding was species specific in that it did not occur with bovine endothelial cells.

In a screen of many human cells lives infected erythrocytes bound to amnionic epithelial cells and a line of apigmented melanoma cells. Binding of *Aotus* infected erythrocytes to human endothelium could be blocked with immune sera from repeatedly infected *Aotus*.

2. Two monoclonal antibodies were identified that bound over the surface of *P. knowlesi* merozoites, caused agglutination and blocked invasion. The antibodies immunoprecipitated a 250,000MW protein and cross reacted with a Philippean and Malaysian strain of the parasite.

3. Variant antigens on the surface of *P. knowlesi*-infected erythrocytes are detected by agglutination of infected cells. Parasitized erythrocytes did not agglutinate with immune sera after passage in splenectomized monkeys including sera from animals previously infected with non-agglutinating parasites. In addition to loss of agglutinability, the parasites passaged in splenectomized monkeys exhibited decreased virulence. Thus the function of the variant antigen may be the evasion of a spleen dependent immune response independent of its ability to vary in response to antibody.

4. We have attempted to identify a protein antigen of SI-RBCs from different SICA variants which could be labeled by cell surface radiolabeling probes and immunoprecipitated only by sera which agglutinate the appropriate variant. Initial studies which indicated that SICA-agglitinability is destroyed by mild trypsin treatment suggested that the antigen(s) (whether composed of amino acid and/or carbohydrate residues) were present on cell surface proteins. Although we have used a variety of radiolabeling probes with different reaction specificities for proteins/glycoproteins and high titer agglutinating sera we have not yet identified a new antigen with the expected specificity of the immunoprecipitation. Parasite antigens have also been radiolabeled using amino acid and carbohydrate precursors and similarly immunoprecipitated after detergent solubilization. These studies have also failed to resolve a SICA antigen after analysis of immunoprecipitated protein on one or two-dimensional gels.

In the course of our work attempting to radiolabel and precipitate the SICA antigen(s) we have identified several other antigens of interest. There are new cell-surface proteins of Mr 180,000, 150,000, 132,000 and 72,000 on SI-RBCs from SICA(+) and SICA(-) *P. knowlesi* parasites that are labeled by lactoperoxidase catalyzed radioiodination. All of these components are removed from the surface of intact labeled cells by brief trypsin treatment. On immunoprecipitation of radioiodinated samples, antigens of Mμ 97,000, 72,000, 65,000 and 45,000 are identified with a panel of sera. The new higher Mr proteins are not precipitated.

By using pyridoxal phosphate/NaB^3H_4 treatment of SI-RBCs we labeled many new proteins via their lysyl residues that were absent from uninfected cells. However, it was not possible to achieve cell surface specific labeling via this labeling method. A protein of Mr > 300,000 was identified from several

SICA variants which has only immunoprecipitated using sera against the same strain of P. knowlesi. This may represent a strain specific antigen.

5. The most important development for continuation of these studies on cell surface antigens of SI-RBCs of P. knowlesi has been clonning of SICA(+) and SICA() parasites, coupled with the introduction of a new method for cryopreservation of cloned parasites. We should now be able to simultaneously thaw different parasite clones and perform biosynthetic and cell surface labeling of these different clones in parallel. Formerly it was only possible to use parasites derived from infected rhesus monkeys and perform one labeling and immunoprecipitation analysis at a time.

Proposed Course:

I. Sexual Stages

1. Study gametocytogenesis in cloned lines of P. falciparum particularly to determine the stability of gametocyte production in such lines and the variations between them; to determine the effect of different growth conditions, i.e.. rapid growth versus stationary growth phase cultures on these properties.

2. Develop the capability of infecting mosquitoes from P. falciparum gametocytes grown in culture.

3. Use cultures of P. falciparum gametocytes to study

- i) synthesis of antigenic components during gametogenesis by metabolic labelling with ^{35}S methione.
- ii) define surface antigens on gametocytes and gametes using ^{125}I labelling techniques.

4. Use sera from animals immunized with sexual and asexual stages of P. falciparum and human sera of individuals exposed to endemic malaria to identify antigens recognized by such sera on sexual and asexual stages of the parasites.

5. Develop monoclonal antibodies against P. falciparum to permit precise characterization of these antigens.

6. Further define the cellular and antigenic targets and mechanisms of transmission blocking immunity in P. gallinaceum:

- i) by using immune sera whose cellular targets have been determined in membrane feeding experiments to isolate antigens from radiolabelled midgut stages of the parasite
- ii) by immunization of animals with specific components on the surfaces of midgut stages precipitated by appropriate sera.
- iii) by development of monoclonal antibodies to such components
- iv) by studying the effects of immune sera and monoclonal antibodies independently on male or female gametes to determine which is the target in the inhibition of fertilization
- v) by using Fab fragments of immune sera and monoclonal antibodies.

7. Explore the relationship between antigens on sexual and asexual stages of malaria and the immunity to each stage:

- i) by testing the effect of sera from animals immunized with components precipitated from sexual stages by monoclonal antibodies on the development of asexual parasites in passive transfer experiments and merzoite invasion assays.

ii) by further studying the effects of infection on the elaboration of antibodies to sexual stages.

iii) By direct comparison of the antigenic components of sexual and asexual stages using various labelling techniques followed by immune precipitation with monoclonal antibodies and immune sera and antigenic competition between preparations of the different stages.

8. Determine the relationship between sexual and asexual immunity to *P. knowlesi* malaria. Is immunity stage specific?

II. Asexual Parasites

1. Attemptimg to identify monoclonal antibody that will block invasion by binding to receptor on merzoites. The immunization will use partially purified antigen (150,000D protein from merzoite surface), use adjuvants such as pertussis or saponin and screen by IFA and assay of uncloned ascites for blocking antibody. If identity blocking monoclonal, then make Fab' fragments to determine reception blocking vs. agglutination. If monoclonal antibody found against merzoites, then isolate and characterize functional components.

2. Study erythrocyte receptor for *P. falciparum* with antibodies to determinants on erythrocytes.

3. *P. falciparum* infected erythrocytes and attachment to endothelium.

a.) Study effect of immune sera or sugars on blocking attachment to endothelium.

b.) Study relation between presence of knobs on *P. falciparum* and attachment. Do some knobbed parasites not bind and thus are missing a critical determinant for attachment?

c.) Find a continuous line to replace human endothelium for the assay. Using antibody and various parasite lines, identify unique determinant on knobs responsible for binding.

4. *P. coatneyi*/*P. fragile* - rhesus model. Develop assay for attachment to endothelium, identify sera that block attachment and determine effects of these sera on developing parasite *in vivo*. If infused during ring stage of this synchronous infection, will the schizonts not sequester and be destroyed in the spleen?

5. Study non-specific immunity in primate malaria. Animals immune to *P. knowlesi* will be infected with *P. coatneyi* and rechallenged with *P. knowlesi*. Will specific clearence of *P.* knowlesi produce non-specific clearence of *P. coatneyi*?

6. *P. knowlesi*/antigenic variation.

a.) Biology and Immunology

i) Does the cloned variant positive parasite (SICA(+)) undergo antigenic variation?

ii) Does the cloned parasite lose the variant antigen (SICA(-)) on passage in splenectomized animals?

iii) Is there a difference in virulence between SICA(+) and SICA(-) parasites. If so, will animals previously infected with SICA(-) be immune to challenge with SICA(=)? Does the variant antigen suppress induction of immunity for vaccination, (i.e. and immunosuppressive molecule)?

iv) Evaluate cellular and antibody function after infection with SICA(+) and SICA(-) parasites.

7. Experiments which indicated susceptibility of the SICA antigen to trypsin treatment will be extended using a variety of proteins and exo/endoglycosidases,

in order to determine whether amino acid and/or carbohydrate structures are part of the SICA antigenic determinant. Periodate oxidation will also be performed to oxidise carbohydrate residues and test the effect of agglutination.

8. of our failure to date to identify a protein antigen with SICA immunoprecipitation specificity we will perform analysis of glycolipid antigens derived from SI-Rbcs of different SICA(+) variant type and SICA( ) cells. New methods have recently been developed to test the antibody levels in polyspecific sera to glycolipid antigens. If anti-glycolipid antibodies are detected, a detailed analysis of the antigenicity of separated glycolipids of SI-RBCs will be performed.

9. In order to enrich our antigenic source of SICA antigens for immunoprecipitation analysis we wil begin our studies on subcellular membrane fractionation from SI-RBCs. Considerable effort will be required to develop methods for rupture of SI-RBCs, membrane purification and then membrane fractionation, especially as only a limited number of members are known for their different membranes within SI-RBCs.

10. Surface membrane radiolabeling studies on cloned K+ and K strains of P. falciparum will be continued in order to identify antigenic determinants associated only with the surface of K+ cells. Such determinants would be potentially involved in the sequestration of SI-RBCs of this parasite in the venous capillaries via attachment of 'knoles'(on K+ but not on K parasites) to endothelial cells. We also plan to compare the biosynthetically labeled proteins and glycoproteins of K+ and K parasites by 2-dimensional electrophoresis. Differences revealed in the total labeled proteins may indicate why K parasites do not express knobs on their surface. For example, if there is a higher molecular weight precursor of components destined for the knob within K parasites we would attempt to identify a defect in insertion of this precursor into the surface membrane.

Significance of Biomedical Research:

The merozoite has been the focus of research for many years because it is the stage that invades red cells and thus is exposed to blocking antibody. Antibodies may block invasion by specifically binding to receptors at any point in the invasion sequence or by agglutinating merzoites. Of the multiple determinants on the merozoite surface, we are attempting to identify and isolate those involved in red cell attachment and those which may be targets for agglutinating antibody.

The membrane of the infected red cell, another target of the immune response, has gained malarial antigens which undergo changes in antigenicity during the course of infection and function to attach P. falciparum infected erythrocytes to endothelium. Recrudesence of parasitemia is associated with new variants. In addition, the red cell membrane has reduced amounts of its normal such as spectrin, band 3 and other glycoproteins. In order to understand how these changes influence the survival and eventual elimination of the parasite, we are attempting to define components in the membrane of host origin and those malarial components exposed to the surface including the variant antigen. These studies may influence the choice of antigens for immunization and may suggest new chemotheraputic approaches.

Whereas the asexual parasites described above are responsible for disease, the gametocytes infect mosquitoes and transmit the infection to others. Characterization of antigens on the surface of gametes should facilitate vaccine development.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00185-02 LPD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pathogenic protozoa: Structure, Virulence factors and endogenous viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project involves study of the biology of the parasitic protozoa, Trichomonas vaginalis, Entamoeba histolytica and Giardia lamblia. The major emphasis for the past year has been on Trichomonas with colloborators Honigberg, Spence, Gupta and Frost. Our laboraotry is in the process of evaluating virulence assays in newborn mice and tissue culture which results will be compared with an adult mouse assay (Honigberg) and with clinical and histopathological pictures in patients (Spence, Gupta) produced by a series of new isolates of T. vaginalis (Spence).

We have investigated several properties of metronidazole-resistant strains of Trichomonas vaginalis: time-dose relationships, pH optima, and susceptibility to hexosamines (Mattern, Natovitz, Spence). Some of these studies have led to the sucessful management of 4 cases of drug-resistant Trichomonas at the Johns Hopkins Hospital (Spence, Mattern).

A systematic laboratory study of induction of metronidazole resistance in T. vaginalis is currently underway to evaluate factors contributing to the induction of drug resistance.

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Summary of Work: (cont.)

Preliminary studies indicate the release of a cytotoxic factor by trichomonads which are actively growing, but not found in extracts of the organism. A systematic search for cytotoxins is in progress. (Mattern, Natovitz)

Assays of virulence of *Trichomonas vaginalis*:

1. Newborn mouse subcutaneous assay: To date the best *in vivo* assay for *Trichomonas* virulence is in the subcutaneous adult mouse assay of Honiberg *et al*. We have employed a subcutaneous assay in newborn Swiss and Balb/C mice since the former assay requires multiple time-consuming measurements of abscess size, whereas the latter requires only a visual inspection. Abscesses develop in the subcutaneous, infrascapular site of inoculations. In the newborn Swiss mouse, employing a relatively avirulent strain maintained in axenic culture for years, abscesses frequently develop, enlarge, rupture, and recur at the same site, the cycle requiring one or more months. We have isolated viable trichomonads from animals injected 15 months previously, but they do not appear to be appreciably increased in virulence; chronic infection in the mouse has been observed for more than 18 months.

Newborn Balb/C mice appear to develop a more acute infection than their Swiss counterparts. The infection consists of a highly necrotizing subcutaneous abscess; the disease is frequently fatal. These animals are being observed for recurrent infection. (Mattern, Natovitz)

2. We are also evaluating a tissue culture virulence assay, similar to that developed for *E. histolytica*, employing baby hamster kidney cells. Cytopathology produced by *T. vaginalis* is dose dependent and different isolates vary considerably in the time required to destroy the cell sheet. This test is being performed on new isolates of *Trichomonas* and will be compared with the adult mouse assay being performed in Dr. Honiberg's laboratory. (Mattern, Natovitz, Daniel)

3. We have preliminary evidence that *T. vaginalis*, when growing in culture with or without mammalian cells, produces a substance which causes a cytopathic effect (CPE) on BHK cells. This appears to be unrelated to the known contact dependent cytopathology of trichomonads but to date has eluded detection in cell-free extracts of *T. vaginalis*. This study is being pursued with new isolates of *T. vaginalis* which have and extraordinary capacity to produce a CPE in BHK cells (Mattern, Natovitz, Daniel).

4. In conjunction with the collaborative study on *Trichomonas* virulence (NIH, U.Mass., Johns Hopkins U.) we are also evaluating certain other characteristics of new *Trichomonas* isolates: Size distribution, growth rate, and stickiness (capacity to adhere to the growth vessel wall). (Mattern, Natovitz, Daniel).

Studies on Metronidazole Resistance in *Trichomonas*:

1. Initial attempt to induce or increase resistance to metronidazole in *Trichomonas* revealed that a susceptible strain which could survive 8μg/ml in a two day exposure assay could not survive and be passaged at 0.5μg/ml. A resistant strain which survived 512 μg/ml likewise succumbed on second passage at 8μg/ml and on third passage at 4μg/ml. A relatively resistant strain which survived 32μg/ml for two days also succumbed in third passage at 2μg/ml. Furthermore, it has been known that resistant strains are resistant

only under "aerobic" conditions and under strictly anerobic conditions (Gas Pak) they are as susceptible as non-resistant strains. In view of the known inhibition of T. vaginalis at the normal vaginal pH of 4.5; growth curves at .5 unit increments from 4.5 ot 8.0 were performed on 3 metronidazole resistant strains. Growth was completely inhibited at the pH extremes and optimal at pH 6.0 as has been found by others with susceptible strains.

Four clinical isolates from patients apparently refractory to therapy with metronidazole were subjected to assay for drug resistance. The minimal inhibitory concentration (MIC) with a standard 2 day exposure to drug, followed by removing the drug and refeeding for viability determination, ranged from 64 to 512μg/ml (16μg/ml for susceptible strains). Two or 3 separate assays (10 total) were performed on the 4 strains. Organisms were exposed aerobically to varying drug concentrations for from 1 to 5 or 6 days and then refed to determine viability. The MIC after 5 or 6 days was 2.5% (1.6 - 6.3%) of the MIC found after 1 day of exposure. This represents about a 2-fold decrease in MIC/day of exposure of Trichomonas to metronidazole. (Mattern, Natovitz, Spence)

Thus it appeared that resistant organisms could be killed by prolonged exposure to doses which kill susceptible strains with short exposure, under anaerobic conditions, or acid pH.

2. These studies have led to the sucessful therapy of the 4 patients (Johns Hopkins Hospital) from whom the resistant isolates were obtained. They were treated for 8 days with intravenous metronidazole (lgm q 4h) and methenamine mandelate (.5g q 6h) to eradicate the parasite from both the genital and urinary tracts and with ascorbic acid (4g. daily) since excess vitamin is readily excreted in the urine and it has reducing properties and may also acidify the urine. These patients have been free of symptoms and free of Trichomonas by culture for from 1 to 4 months post therapy. (Spence, Mattern)

Brugia Malai An ultrastrucural study has been initiated to elucidate the interaction of human leukocytes and microfilaria. Progressive degeneration changes occurred in unsheathed microfilaria exposed to leukocytes from 2 to 15 hr. whereas sheathed microfilaria appeared unaffected. (Daniel, Weil).

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Spence, M.R. and Mattern, C.F.T. Management of metronidazole-resistant *Trichomonas vaginalis* infection. Morbidity and Mortality Weekly Report (submitted)

Honors and Awards:

Dr. Mattern serves on the editorial board of the Journal of Protozoology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00197-02 LPD

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immune Recognition in Filariasis and Other Helminth Infections

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Rabia Hussain	Collaborative Researcher (IPA)
Other:	Eric A. Ottesen	Senior Investigator, LPD, NIAID
	Nuzhat Anwar	Visiting Fellow, LPD, NIAID
	Theodore E. Nash	Senior Investigator, LPD, NIAID

COOPERATING UNITS (if any)

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NIAID, Bethesda, Maryland 20205

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18/12		

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of this project is to characterize host immune responses in filariasis and relate the findings to pathogenesis and/or protection. Quantitative and qualitative aspects of IgE responses are being rigorously characterized. Highly sensitive radioimmunoassays and crossed radioimmunoelectrophoresis have been developed to identify filarial allergens to which the IgE anitbodies' response is directed. In addition, IgG responses are also being quantitated and their importance in blocking IgE-mediated responses is under investigation.

Characterization of antigens from different stages of the parasite (infected larvae, L3, and microfilaria) as well as excretions and secretions and surfaces is being carried out using immunochemical techniques to identify unique and common antigens. Such information should prove useful in developing immunodiagnostic reagents and also identify antigens important in disease protection.

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Project Description:

Humoral immune responses to helminth infection are being studied with particular emphasis on IgE responses in filariasis and to correlate these responses with the wide spectrum of clinical presentations observed in endemic populations.

Quantitation of filaria specific IgE and IgG levels in a bancroftian endemic population. We have previously reported quantitation of IgE in a Cook Island population using a highly sensitive radioimmunoassay. We have now extended these studies to an India population in Madras, India.

Eighty-three sera, representing the entire clinical spectrum were quantitated for their IgE responses. The highest filaria specific IgE was observed in patients with tropical pulmonary eosinophilia (8630 ug/ml), and this level was significantly different (p 0.001) from all other groups. Patients with circulating microfilaria but no other clinical signs showed the lowest levels (31 ug/ml) and those with chronic lymphatic blockage had slightly higher levels (68 ug/ml). Endemic controls showed considerable levels of IgE also (55 ug/ml). These levels are considerable when compared with pollen-allergic individuals. However, this antibody accounted for less than 1% of total IgE in microfilaremics and between 0.2-15% (mean 2.5%) in individuals with chronic pathology indicating the tremendous polyclonal activation of nonspecific IgE. Interestingly, the only group in our study which showed high percentages of filaria-specific IgE was the subset which exhibited allergic symptoms where filaria-specific IgE account for 12-48% of the total. These percentages are very similar to those reported for pollen allergic individuals. Thus, high percentages of filaria-specific IgE may be important for onset of allergic symptoms.

A. Qualitative characterization of IgE responses species-specificity of IgE responses. There has been some suggestion that there may be greater antigen specificity in the IgE response than in the IgG or IgM responses. This question was directly asked in our laboratory by using homologous and heterologous antigens. There was much less cross-reactivity with heterologous parasite in the IgE system than the IgG system. This was further confirmed in ten individual sera where greater amount of IgG antibodies could be absorbed out by heterologous antigen sorbent than the IgE antibody. The species-specific antigens to which these IgE antibodies are directed is being further investigated.

B. Identification of antigens (allergens) in filariasis. Antigens which are capable of inducing and binding to the IgE class of antibodies are referred to as allergens. Allergens are often proteins or degradation products of proteins with molecular weights 10,000 - 40,000 and pI 4-6. Consequently, they are well suited for analysis in crossed immunoelectrophoresis at ph 8.6 by means of precipitating antibodies raised in animals. Among the number of precipitates formed, it will be possible to demonstrate those which are able to bind patient serum IgE by means of radiolabeled anti-IgE followed by autoradiography. This method referred to as crossed radioimmunoelectrophoresis (CRIE) was first introduced to identify allergens in Timothy grass extracts and later extended to ragweed pollen extracts. We have used CRIE to identify the Brugia malayi antigens binding IgE antibodies in filariasis sera. High titered precipitating antisera were raised in rabbit to soluble Brugia malayi adult antigens. This antiserum could recognize over 30 antigens in crossed immunoelectrophoresis. Following the formation of precipitates, the slides were washed and incubated with either normal serum or a filariasis serum pool. The unreacted serum proteins were washed and the slide was further incubated with radiolabeled anti human IgE (F_c specific). After washing and drying, autoradiography was performed by exposing Kodak X-ray film to dried gel for varying lengths of time. Normal serum showed very little binding of anti-IgE. However, several radioactive peaks were observed on the plate incubated with filariasis serum pool. In fact, most of the antigens were being recognized by the IgE antibodies. This is in sharp contrast with the pollen allergen system where only a limited number of antigens recognizes IgE antibodies. Thus, it seems that parasite infections are unique in terms of not only deregulating the nonspecific IgE synthesis (polyclonal activation) but also antigen-specific IgE synthesis.

This system of CRIE can now be used to further ask the questions: is recognition of antigens by the IgE different in different groups? Does recognition of different patterns of antigens influence pathogenesis? This system can also be further utilized in conjunction with radioallergosorbent techniques to identify species-specific antigens being recognized by the IgE system and would also aid in identification and purification of such antigens.

C. Excretory-secretory antigens usually are more species specific and less complex than somatic antigens. These antigens are being rigorously characterized (Anwar) and both IgE and IgG responses to these and other stage-specific antigens are being further studied. The uniqueness of antigens present in each of the various stages is being studied immunochemically using radioimmunoelectrophoresis and SDS polyacrylamide gel electrophoresis in combination with autoradiography.

Publications:

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2. Hussain, R., Norman, P.S., and Marsh, D.G.: Rapidly released allergens from short ragweed pollen. II. Identification and partial purification. J. Allergy Clin. Immunol. 67: 217-222, 1981.

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4. Hussain, R., Hamilton, R.G., Adkinson, N.F., Kumaraswami, R., Ottesen, E.A.: The IgE response in human filariasis. 1. Quantitation of filaria specific IgE. J. Immunol. (in press).

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6. Freidhoff, L.R., Meyers, D.A., Bias, W.B., Chase, G.A., Hussain, R., and Marsh, D.G.: A genetic-epidemiologic study of human immune responsiveness to allergens in an industrial population. I. Epidemiology of reported allergy and skin-test positivity. Am. J. Med. Gen. (in press).

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8. Hendricks, A.A., Calvin, L., Elfenbein, G.J., and Hussain, R.: Erythema annulare centrifugum associated with ascariasis. Arch. of Dermatology (in press).

9. Lowenstein, H. King., T.P., Goodfreind, L.G., Hussain, R., Roebber, M., and Marsh, D.G.: Antigens of Ambrosia elatio (short ragweed) pollen. II. Immunochemical identification of known antigens by quantitative immunoelectrophoresis. J. Immunol. (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00198-02 LPD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Host Immunological Response to Giardia lamblia Infection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P.D. Smith	IPA, University of Colorado Medical Center, Denver, Colo.
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T.E. Nash	Senior Investigator, LPD, NIAID
F.D. Gillin	Senior Staff Fellow, LPD, NIAID
D.B. Keister	Biologist, LPD, NIAID
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2	6	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project has been to characterize the host immunological responses to infection with G. lamblia. We initially examined giardiasis patients for the presence of circulating anti-G. lamblia antibodies by an enzyme-linked immunosorbent assay. IgG anti-G. lamblia antibodies were detected in serum. We will examine body secretions for IgA anti-G. lamblia antibodies. The immunoreactive antigens to which these antibodies are directed were characterized.

To examine the cellular immune responses in giardiasis patients, we are studying in vitro lymphocyte transformation in response to G. lamblia trophozoite antigen. The effector mechanism of cellular interaction with G. lamblia has been investigated with spontaneous cell-mediated and antibody-dependent cytotoxicity assays. Characterizing the humoral and cellular immunological responses in patients with giardiasis may elucidate how host defense mechanisms modulate giardiasis.

Project Description:

There is mounting epidemiolgical, clinical, and experimental evidence that immune factors are important in host response to this parasite: the epidemiological evidence from Colorado suggests that repeated exposure may confer partial protection against G.lamblia. The clinical observations that 80% of U.S. patients with immunoglobulin deficiencies and diarrhea have giardiasis supports a role for humoral immune factors in host response to G. lamblia. Finally, the experimental evidence that nude mice are more susceptible to Giardia muris suggests cellular immune factors may also be functional in host defense against giardiasis.

These immune factors may contribute to the variable clinical course of subsets of patients with giardiasis. These subsets include asymptomatic carriers, acute symptomatic carriers who respond to antibiotic therapy, chronic sympotmatic carriers with normal immunoglobulin levels and chronic symptomatic carriers with depressed immunoglobulin levels. The following projects have been established to investigate the role of humoral and cellular immunity in modulating host response to G. lamblia infection.

1. Parasite Antigen. G. lamblia tropnozoites were isolated from a patient with chronic giardiasis. To investigate the mechanism of this patients' diarrhea and his immune response to his infection, we isolated and cultured his organism and examined a) parasite drug sensitivity; b) possible enterotoxin production by the parasite; and c) the patients' humoral and cellular immune responses to the organism (manuscript submitted). This G. lamblia trophozoite is the organism employed in all subsequent investigations.

2. Antigen Characterization. We have completed initial studies to immunochemically characterize the immunoreactive antigens in G.lamblia to which human antibody is directed. Employing SDS polycrylamide gel electrophoresis, immunoelectrophoresis, crossed immunoelectrophoresis and an enzyme linked immunosorbent assay (ELISA), subtle but potentially important antigenic differences were identified (manuscript in preparation).

3. Humoral Antibody Response. In completed work we demonstrated by an indirect immunoflorescence antibody test the presence of serum anti-G. lamblia antibodies in 30 patients. This work was extended using a more objective ELISA which allowed us to confirm the presence of systemic antibody and show it to be of the IgG class. High antibody titer does not appear to be protective against chronic or recurrent giardiasis suggesting that the quantity of antibody is not protective.

4a. Cellular Immune Response. To assess the cellular immune response, we have established an in vitro lymphocyte transformation assay using sonicated G. lamblia trophozoites as antigen. Lymphocyte activation in individuals with giardiasis is being studied.

4b. Cytotoxicity for G. lamblia. To study the mechanism of cellular interaction with G. lamblia, a cytotoxicity assay has been established. Employing peripheral blood lymphocytes incubated with ^{3}H-thymidine labeled

G. lamblia we have demonstrated a high degree of spontaneous cell-mediated cytotoxicity for G. lamblia in uninfected individuals. The majority of this effector cell activity is in the monocyte-macrophage fraction (manuscript submitted). We are investigating spontaneous monocyte cytotoxicity for G. lamblia in giardiasis patients.

In a second series of experiments, we have shown antibody dependent cell-mediated cytotoxicity (ADCC) of gramulocytes for G. lamblia. This effector activity is present in both polymorphonuclear leukocytes and eosinophils and is not complement mediated. Deficiency of ADCC in hypogammaglobulinemics may contribute to the high incidence of giardiasis in this subset of patients.

Publications:

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Smith, P.D., Gillin, F.D., Spira, W.M. and Nash, T.E.: Chronic giardiasis: studies on drug sensitivity, toxin production and host immune response. Manuscript submitted.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00207-02 LPD

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immunity and Immune Evasion in Parasitic Infection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	S. L. James	Guest Worker, LPD, NIAID
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	T. McCutchan	Staff Fellow, LPD, NIAID
	F. Gillin	Expert, LPD, NIAID
	M. Crane	Visiting Fellow, LPD, NIAID
	M. Meltzer	Senior Investigator, LIB, NCI
	J. Lazdins	Collaborative Researcher, (IPA), LIB, NCI
	D. Singer	Staff Fellow I, NCI

COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major goals of this project are to identify mechanisms of protective immunity against parasitic infections while at the same time studying mechanisms by which parasites evade the immune response. In schistosomiasis, these studies involve the identification of effector mechanisms by which schistosomula are killed in mice with either concomitant or irradiated cercarial vaccine-induced immunity to challenge infection. In particular, the role of activated macrophages in schistosomulum killing has been closely analyzed. Schistosomes were also studied in terms of their ability to evade host immune responses and the possibility that they actively mimic the host, analyzed by assaying the genome of the parasite for hybridization with host total DNA as well as cDNA probes. In another study, an approach to immunization against parasites employing anti-idiotype antibodies was explored, and in preliminary experiments the injection of these antibodies was found to partially protect mice against a variant of African trypanosomiasis. The role of the alternative complement pathway (ACP) as a defense against parasitic infection was studied with the protozoa Trichomonas vaginalis and Trypanosoma cruzi, and a millipore chamber implantation technique was developed for inducing the transformation of ACP sensitive epimastigotes into ACP resistant trypomastigotes in mice.

Project Description

A. Schistosomiasis

Effector mechansims of immunity against challenge schistosomula were examined in both the irradiated vaccine and concomitant immunity (chronic infection) models of resistance in the mouse. Immunity induced by irradiated cercariae was shown to be thymus-dependent (absent in athymic nude mice) to be species specific (mice immunized with *S. mansoni* fail to reject *S. japonicum*) (Cheever, Sher) and to be induced preferentially by early (cercariae and newly transformed schistosomulae) as opposed to later (lung) larval stages. A mouse strain survey was performed and a polymorphism studied in the levels of immunity developed by inbred mice after vaccination. The development of high resistance was shown to be inherited as a dominant trait and to be unlinked to the mouse major histocompatibility complex (Sher and James).

Studies on concomitant immunity (James, Lazdins, Meltzer) focused on the role of the macrophage as an effector cell in schistosomulin rejection. It was demonstrated that after primary infection with *S. mansoni* mice develop highly elevated peritoneal cell numbers with most of these cells being macrophages. The cells were shown to be activated macrophages as judged by their ability to lyse radiolabeled tumor cell targets. In addition, these cells were demonstrated to be capable of killing schistosomula *in vitro*, arguing that activated macrophages may also play a role in concomitant immunity to *S. mansoni in vivo*.

Work was also initiated on the characterization of schistosome nucleic acids with the aim of studying relationships between the genomes of the host and parasite and with the longer range goal of cloning specific genes involved in protective immunity (Simpson, McCutchan, Singer). Lysis of cercariae in SDS followed by phenol/chloroform extraction procedures resulted in the satisfactory recovery of genomic DNA from the parasite. When adult worms were similarly treated, however, large amounts of carbohydrate were extracted with the nucleic acids. A cesium chloride gradient centrifugation was employed to separate the DNA from the carbohydrate and render the former sufficiently pure for further analysis. Initially, liquid hybridization studies were utilized to characterize the parasite genome. The renaturation profile of adult schistosome DNA demonstrated that approximately 40% of the genome consists of moderately to highly repeated sequences of DNA and that the size of the genome is 0.27×10^{-12} g/cell or approximately 270×10^{6} base pairs. Hybridization between adult and cercarial DNA demonstrated that nucleic acid from the two sources was indistinguishable. Thus, no significant acquisition of host DNA during inactivation of the parasite is evident. This was further confirmed by hybridization of adult DNA with mouse (Balb/c) DNA. No sequence homology was detected. To assess the possibility of discrete homology between parasite and host not demonstrable by CoT analysis, specific gene hybridization was carried out. No hybridization between cytochrome C, dihydrofolate reductase or β-actin was observed.

Interestingly, however, a cDNA to human HLA that hybridized to mouse H-2 was found to also hybridize with the parasite genome. We have shown that the pattern of hybridization is the same with cercarial and adult DNA, but different with mouse DNA and is thus unlikely to represent host contamination. The hybridization indicates the presence of several genes within the parasite genome that have homology with genes in the mammalian major histocompatibility complex.

B. Protozoa:

African and American Trypanosomiasis, Trichomoniasis. The first project on protozoa involved the development on a new approach for inducing protective immunity against parasites by the use of anti-idiotype antibodies (Sacks). Control of African trypanosome infections in the mouse is mediated by antibody specific for the variant-specific surface antigen of the parasite. Monoclonal antibodies raised against one such antigen by Klaus Esser at Walter Reed will passively protect a mouse against infections by cloned T. rhodesiensi trypanosomes of the homologous antigenic type. We have successfully raised mouse antibody specific for the idiotypic determinants of each of three such monoclonal reagents, and we have been attempting to induce the expression of these idiotypes by injection of the anti-idiotypic antibody. In a preliminary experiment, injection of 1 ug of the IgG_1 fractions of all three anti-idiotype antibodies 5 weeks before challenge with a low infective dose of trypanosomes, resulted in four of ten mice being completely protected. The protection seen in these mice was correlated with their having a significant enhancement in the expression of two of the three idiotypes. The levels of these idiotypes expressed in response to control infections varied considerably between individual mice, but in no case did they approach the levels seen in the protected animals.

In a recent experiment, 1 ug of anti-idiotypic antibody again appeared to induce optimal effects in terms of protection. However, in those receiving either 200 ng or 5 ug of anti-idiotypic antibody, there was a significant increase in the number of infected mice whose first wave parasites no longer expressed the variant antigen of the infecting clone.

This work demonstrated that idiotypic manipulations by anti-idiotypic antibody, which has proven so successful in those systems where the idiotype in question is a dominant one, can also be effective for idiotypes expressed in low frequency. More importantly, it is the first demonstration that anti-idiotypic antibody can be used as an antigen substitute in order to induce immunity to a microbial pathogen.

The second major project on protozoa concerned the role of the alternative complement pathway (ACP) as a natural defense against parasite infection and the mechanisms used by protozoa in evading ACP-mediated lysis. In one study (Gillin, Sher) the lysis of Trichomonas vaginalis in fresh normal serum was studied and shown to be the consequence of the activation of the ACP. No evidence could be obtained for the contribution of the classical pathway in the serum-mediated lysis of the parasite.

In studies on T. cruzi, we (Crane, Sher) asked what the effect of implantation in the host would be on epimastigotes which in vitro are lysed by the ACP. Epimastigotes of the Tulahuen strain were inoculated into 0.45 μ millipore chambers which were then implanted subcutaneously into mice. When recovered 24 hours later, the parasites were found to have converted to trypomastigotes which in their morphology more closely resembled blood stream as opposed to metacyclic stage trypomastigotes. The converted trypomastigotes were found to be totally resistant to ACP-mediated lysis. Kinetic experiments revealed that the transition in sensitivity to C lysis preceded the transformation into trypomastigote, suggesting the existence of a C-resistant epimastigote intermediate. Transformation from epimastigotes into trypomastigotes was shown to be dependent on the strain of T. cruzi employed, fast growing strains such as Tulahuen and Sylvio transforming while a slower growing strain, NIH-Y, does not transform. Transformation was also shown to be dependent on the growth cycle of the parasite, occurring with log phase parasites but not with stationary phase organisms.

That the ACP is potentially active against epimastigotes in vivo was suggested by the observation that Tulahuen epimastigotes implanted into guinea pigs (which have a more potent C system than mice) are recovered in smaller numbers than from mice and recovered exclusively as trypomastigotes. In contrast, the NIH epimastigotes, which as mentioned earlier fail to convert to trypomastigotes in mice, are completely destroyed inside chambers implanted in guinea pigs.

Experiments are now being planned to analyze the chemical basis of the surface change responsible for the transition from C-sensitive to C-resistant parasites.

Publications:

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2. Samuelson, J.C., Sher, A., and Caulfield, J.P.: Newly transformed schistosomula spontaneously lose surface antigens and C3 acceptor sites during culture. J. Immunol. 124: 2055-2057, 1980.

3. James, S.L., and Sher, A.: Immune mechanisms which stimulate leukocyte migration in response to schistosomula of Schistosoma mansoni. J. Immunol. 124: 1837-1843, 1980.

4. Brown, A.P., Burakoff, S., and Sher, A.: Specificity of alloreactive T lymphocytes which adhere to lung stage schistosomula of Schistosoma mansoni. J. Immunol. 124: 2516-2518, 1980.

5. Moser, G., Wassom, D.L., and Sher, A.: Studies of the antibody dependent killing of schistosomula of Schistosoma mansoni employing haptenic target antigens. I. Evidence that the loss in susceptibility to immune damage undergone by developing schostosomula involves a changes unrelated to the masking of parasite antigens by host molecules. J. Exp. Med. 152: 41-53, 1980.

6. Sher, A., and Moser, G.: Schistosomiasis: Immunological properties of developing schistosomula. Presidential Symposium, American Association of Pathologists, 1980. Am. J. Path. 102: 121-126, 1981.

7. Moser, G., and Sher, A.: Studies of the antibody dependent killing of schistosomula of Schistosoma mansoni employing haptenic target antigens. II. In vitro killing of TNP-schistosomula by human eosinophils and neutrophils. J. Immunol. 126: 1025-1029, 1081.

8. Abbas, A., James, S.L., and Sher, A.: Immunogenicity of haptenated schistosomula in vitro. J. Immunol. 126: 1022-1024, 1981.

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13. Sher, A., and Benno, D.: Decreasing immunogenicity of developing schistosome larvae. Parasite Immunol. In press.

14. Caulfield, J.P., Hein, A., Moser, G., and Sher, A.: Light and electron microscopic appearance of rat peritoneal mast cells adhering to schistosumula of Schistosoma mansoni by means of complement or antibody. J. Parasitol. In press.

15. Miller, K.L., Smithers, S.R., and Sher, A.: The response of mice immune to *Schistosoma mansoni* to a challenge infection which bypasses the skin: evidence for two mechanisms of immunity. Parasite Immunol. 3: 25-31, 1981.

16. Gillin, F.D., and Sher, A.: Activation of the alternative complement pathway by *Trichomonas vaginalis*. Infect. Immun. In press.

17. James, S.L.: In vitro proliferative response to living schistosomula by T lymphocytes from mice infected with *Schistosoma mansoni*. Parasitol. In press.

18. Rumjanek, F.D., and Simpson, A.J.G.: The incorporation and utilization of radiolabeled lipids by adult *Schistosoma mansoni in vitro*. Mol. Biochem. Parasitol. 1: 31-44, 1980.

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20. Simpson, A.J.G., Cesari, I.M., and Evans, W.H.: Isolation and characterization of the tegumental outer membrane of adult *Schistosoma mansoni*. Proceedings of 3rd International Symposium on "Biochemistry of Parasites and Host Parasite Relationships. The host-invader interplay."

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00208-02 LPD

PERIOD COVERED
October 1, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

DNA of P. berghei and S. mansoni

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: T. McCutchan	Sr. Staff Fellow, LPD, NIAID
OTHERS: J. Dame	Staff Fellow, LPD, NIAID
J. Mullins	
A. Simpson	

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

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42/12	30/12	12/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have undertaken two projects aimed at characterization of the genus of P. berghei and S. mansoni. To study the malarial parasite, P. berghei, we are developing a series of polymorphic genetic DNA markers using DNA recobinant technology. These markers will prove useful not only in the study of malarial genetics but also as tools that can be used to distinguish closely related lines of the parasite. To date we have cloned over 1000 individual DNA segments from the P. berghei genome in the E. coli plasmid vector pBr322. We have radioactively labeled some of these recombinant plasmids and have used them as probes in DNA studies using the Southern Blot technique. We have found that 4 of the recombinant plasmids studied give easily recognized patterns of bands when hybridized to genomic DNA from P. berghei. This will make them useful as probes for DNA polymorphism. We have found that the pattern of DNA hybridization varies when genomic DNA from different species of mouse malarial parasites are tested and we take this as an indication that these markers will also be useful in population studies.

We have undertaken theS. mansoni project in collaboration with A. Simpson and A. Sher. We are studying the reassociation kinetics in solution of

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(Rev. 10-76)

Summary of Work (cont.):
DNA from Schistosoma mansoni. We have determined the genome size and content of repetitive DNA sequences of this organism. We have also been able to compare the DNA from adults and cercariae by restriction analysis and the Southern blotting technique. Here we used radioactive DNA probes that are cloned from other eucaryotic systems (eg. actin gene, HLA gene); but which detect related sequences in S. mansoni.

Project Description:

In recent years DNA research has undergone a boom due to the discovery of restriction nucleases and the development of techniques for DNA cloning, DNA sequencing, and Southern blot analysis. With these tools researchers can now study gene structure, the arrangement of genes within the genome, and can compare the genomes of related organisms with new precision. In certain systems, the protein from an isolated gene can even be produced in small amounts. Our intention is to use this technology to investigate 1) rearrangement of genes in a malarial parasite genome during development; 2) changes in gene arrangement as a result of genetic crosses between related organisms and 3) the usefulness of DNA probes to facilitate detection of strain and species differences. We plan to develop DNA probes to use in much the same way as polymorphic proteins have been used in the past to distinguish strains. The differences will be that 1) a great number of polymorphic DNA markers can be quickly generated, 2) that they can all be easily and specifically assayed by DNA hybridization techniques, and 3) that there is never any confusion as to the source of DNA (host or parasite).

Our approach depends on being able to clone specific fragments of malarial DNA in a bacterial vector and produce them in large quantities. We have done this by cutting total P. berghei DNA with the restriction nuclease Bam HI which breaks the high molecular weight DNA at defined points producing a large number of fragments of defined length. These thousands of fragments are then inserted into the plasmid vector pBr322 and the recombinant molecules are transfected into a bacterial host. The transfection is carried out under conditions such that each bacterial colony receives only one recombinant DNA molecule. Thus the large quantities of bacteria grown from an isolated colony contain only a single DNA fragment in very large amounts.

Cloned DNA that is radioactively labeled can be then used to detect complementary DNA (DNA with the same nucleotide sequence) from within a mixture of other DNAs. If one cuts total DNA with a restriction nuclease and separates the fragments according to size by gel electrophoresis, then size or Rf of the fragment complementary to the cloned DNA can be determined by Southern blotting and liquid hybridization techniques. Using this Southern blot technique with several different restriction nucleases which have different cleavage specificities, one can detect small differences in DNA structure within and around the DNA which is complementary to the cloned DNA probe. This technique can detect polymorphism at the DNA level.

Our work has already produced cloned segments of DNA that will be useful as polymorphic genetic markers, and in addition, we have cloned the P. berghei ribosomal genes and a highly repeated DNA sequence that is interspersed in the genome.

Our work on Schistosoma mansoni has taken a somewhat different approach.

Little basic work on the DNA of this organism was published and therefore we started the basic liquid hybridization kinetic experiments to investigate the genome size to determine the content of repeated and single copy sequences. These studies have revealed the S. mansoni genome is 2.7×10^8 nucleotide pairs in size and that almost 40% of the genome is composed of highly to moderately repeated sequences. Further the nucleotide composition is being investigated to look for minor modified bases which might be important in development. This type of study is done by standard chemical and chromatographic techniques and is is important because the presence of modified bases in DNA affects the interpretation of restriction nuclease data. Finally, we are using cloned genes from other organisms as probes to identify similar sequences in the S. mansoni genome and to determine if their structure is similar to that of their counterparts in other organisms. We are interested in whether or not some parasitic genes have been appropriated from the genome of the host.

Publications:

None

LABORATORY OF STREPTOCOCCAL DISEASES
1981 Annual Report
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PHS-NIH
Summary Statement
Laboratory of Streptococcal Diseases
October 1, 1980 to September 30, 1981

Research Progress

Bacterial Lipoteichoic Acid

Lipoteichoic acid (LTA) is a major amphiphilic molecule (a glycerol phosphate polymer with a phosphoglycolipid end group) that is found predominantly associated with the mesosomes (membranous organelles) of Gram positive bacteria. It may have several roles in the growth and physiology of the bacterium, but most of these are not clearly defined; and its physical nature is poorly understood. Recent studies were directed at determination of some of the unique properties of staphylococcal LTA, namely its density, degree of aggregation, and affinity for various surfaces. Agarose-purified LTA-I had a density of 1.3 and a sedimentation constant in sucrose gradients of 8S, a molecular weight of 160,0000, and an aggregate size of 40 LTA monomeric units (assuming MW = 4000 for LTA monomer). In dilute solution (1 ug/ml or less) these 8S aggregates dissociated to 2S aggregates (16,000 MW and 4 monomeric units in size). At higher LTA concentrations (3 ug/ml or higher), aggregates of 11S and 15S (in addition to 8S) were formed. The 8S and higher aggregates of LTA observed on sucrose gradients eluted from agarose as peak I and the 2S aggregates as peak II. The 2S (but not 8S) aggregates absorbed to agarose, leading to losses of peak II (but not peak I) during LTA purification on agarose. LTA was shown to absorb to hydrophobic surfaces, cellulose nitrate and glass-saturating at 5 to 10 nanograms/cm^2. The use of sucrose gradients for the separation of various LTA aggregates led to high recoveries of peak II LTA (2S) not possible on agarose.

Mesosomes from S. aureus were separated for the first time into heavy and light types (density 1.15 and 1.13, respectively). Osmotic rupture and release of LTA from these mesosomes was accomplished, so that it is now possible to measure specific protein-LTA interactions which occur in these organelles. (Huff)

LTA isolated and purified from Staphylococcus aureus, and antisera prepared to the polyglycerolphosphate backbone portion of the molecule, were used to assess the role of this polymer as a carrier of streptolysin S (SLS) produced by Group A streptococci. The activity with LTA as a carrier was equal to or better than that obtained with the usual artificial carrier, RNA-core. Fully acylated LTA was needed for the expression of SLS activity and removal of the fatty acid portion of the molecule (deacylated LTA) resulted in a complete loss of activity, suggesting that this portion of the LTA molecule is involved in the binding of the hemolytic moiety. Added proof of such binding was shown by inhibition of SLS activity when antiserum was added either prior to or during the activation step. The results show that LTA can function as a physiological carrier of SLS, and possibly as a carrier of other protiens. (Theodore and Calandra) LTA has also been implicated in the adhesion of bacteria to tooth surfaces, resulting in plaque formation and dental caries. LTA extracted from dental plaque of monkeys was assessed by passive hemagglutination inhibition. LTA content of plaque fluid was negligible (1:16 titer) whereas, from as little as 50 ug of plaque solid material, a titer of 1:256 was obtained. This method is only semiquantitative, but allows for the detection of minute amounts of LTA from individual plaque samples. Attempts are now being made to quantitate the LTA in plaque by use of rocket immunoelectrophoresis. (Theodore and Ciardi, NCP, NIDR)

Streptococcal Bacteriophages

Because of lack of information in the literature, a comparative study of some properties and nucleic acids of 8 bacteriophages that lyse or lysogenize streptococci of 6 serogroups (A, B, C, E, G, H), was conducted. All phages possessed genomes of linear double-stranded DNA. Six long-tailed phages (all temperate except one, virulent for Group A streptococci, and of obscure origin) had genomes of similar molecular weight (23-25 x 10^6) and G + C content (40-42 mol.%); and all but one (from Group B) demonstrated both circular permutation and terminal redundancy of nucleotide sequences. Four of these (from Groups A, E, and G, and including the virulent phage) showed identical lytic patterns and close serological relatedness; and their DNAs, compared by restriction endonuclease fragmentation patterns, were closely similar. Two virulent phages, infecting streptococci of Groups C or H, had complex short-tailed morphologies, lower molecular weight DNAs (10.6 x 10^6 for C1, 11.4 x 10^6 for H39), and lower G + C contents (37.5 mol.% for C1, 35.2 mol.% for H39): neither genome was terminally repetitious and only one (H39) was circularly permuted. These findings indicate previously unsuspected similarities, in addition to morphology, of long-tailed perdominantly temperate phages of different origins, and emphasize their differences from short-tailed phages that do not lysogenize. Other studies, by lysogenization and prophage curing methods, confirmed that streptococcal erythrogenic toxin B production, unlike toxins A and C, is not coded by a prophage genome. (Moynet, Colon-Whitt, Cole, Popkin; Defilippes, Garon, LBV)

New Morphologically Unique Mycoplasma Discovered

A serologically distinct mycoplasma was isolated, by use of a special medium, from the urethras of 3/14 male patients with nongonococcal urethritis (NGU). The organism is predominantly flask-shaped, possesses a specialized terminal organelle, and is covered over the anterior two-thirds by a prominent outer layer or "nap" by which it adheres to erythrocytes or other cells. It thus resembles in some degree, but is different than, the human respiratory pathogen Mycoplasma pneumoniae and several other morphologically differentiated mycoplasmas found in birds, cows, pigs, and cell cultures. Incidence and pathogenicity of the new isolate, which has not yet been completely defined taxonomically nor named, is being explored in further clinical studies and tests in primates. It should prove a useful tool in investigation of mechanisms of microbial adherence, as well as a probable addition to the spectrum of causes of NGU. (Cole, Tully, Popkin, Rose; Taylor-Robinson, MRC, Harrow)

Ultrastructure and Adhesion of Flask-shaped Mycoplasmas

Those mycoplasmas that hemadsorb and cytadsorb are presumed to do so by virtue of outer layers functionally analagous to pili, fimbriae, or capsules of adhering walled bacteria. Such layers have been clearly defined by cytochemical staining and/or enzymatic removal in some mycoplasmas, but have not been thoroughly resolved in a heterogeneous group of flask-shaped mycoplasmas that also possess differentiated tips or terminal organelles by which they are presumed to adhere. The finding of a new mycoplasma of similar morphology and possessing a prominent outer layer over its anterior end (See above summary) stimulated a comparative electron microscopic study. Mycoplasma pneumoniae was found to possess a previously undetected outer layer on the tip, similar to but more restricted than, that of the new genital mycoplasma: this layer, as well as the terminal organelle, appear to be defective or absent in hemadsorption-negative mutants. Several other species (M. gallisepticum, M. alvi, M. sualvi, M. pulmonis, and a representative of undescribed serogroup 38) possess outer layers of thicknesses, differing by species, that cover the entire mycoplasmal surface. However, the tips of some of these can be distinguished by extremely restricted zones of undefined amorphous material: also, some of them do not have the well-developed striate internal rods that characterize the terminal organelles of the other species. These ultrastructural findings are being correlated with studies of hemadsorption, cytadsorption, and adherence to glass plastic surfaces: in conjunction with biochemical investigations of cell proteins, these experiments may clarify the nature and role of terminal organelles and associated surface structures. (Cole, Tully, Rose, Popkin; Clyde, Collier, Hu, U. North Carolina; Baseman, U. Texas at San Antonio)

Arthritis Induced by Streptococcal Cell Walls in Rats is Genetically Determined

Intraperitoneal injection of sonicated and partially purified cell walls of Group A streptococci results in a chronic symmetrical distal polyarthritis in most outbred rats. The use of 16 inbred strains revealed strain differences in susceptibility to arthritis, and analysis indicated genetic dominance and dependence on multiple genes for arthritis development. A second feature, previously undescribed, was the presence of non-caseating granulomas in liver and spleen; and correlation of the extent of these lesions with severity of arthritis. Preliminary studies indicate that arthritis-susceptible rats (but not others) reestablish cell-mediated immunity lost initially after cell wall injection--suggesting that such immunity is required for development of arthritis. Details of the phenonomena in the rat model are being explored as an aid to further understanding of the mechanisms responsible for arthritis--especially that following streptococcal infections. (Calandra; Wilder, NIADDK)

Tetanolysin Structure and Function

Tetanolysin, an oxygen-labile hemolysin produced by Clostridium tetani, functions like similar hemolysins such as streptolysin O (Streptococcus pyogenes), perfringolysin (Clostridium perfringens), and cereolysin (Bacillus cereus). Though formerly believed to form characteristic arc- and ring-shaped ultrastructure lesions on cell membranes solely as a result of interaction with membrane cholesterol, some toxins (perfringolysin, cereolysin) were recently shown to consist of arcs and rings in purified preparations. Our electron microscopy of highly purified and concentrated tetanolysin, negatively stained or metal shadowed, with or without prior fixation in the absence of lipid, showed the same characteristic arcs and rings of the usual 25-30 nm internal diameter, formed from 4-8 nm subunits. Arc and ring formation appears to be a polymerization dependent on subunit concentration: in dilute preparations exposed to lipid dispersions (artificial liposomes), an apparent affinity of the subunits for cholesterol led to concentrations near or on liposomes, with subsequent formation of visible arcs and rings in proportion to liposome cholesterol content (or, more precisely, to cholesterol-phospholipid ration). The EM findings were consistent with results of studies of tetanolysin binding and of effects of tetanolysin on conductance across black lipid membranes formed from cholesterol. They indicate the importance of critical (threshold) concentrations of both tetanolysin and cholesterol in binding and disturbing the bilayer structure of the liposome, and supply a model for understanding oxygen-labile hemolysin effects on natural cell membranes. (Cole; Rottem, Hadassah; Habig, Hardegree, BOB, FDA)

Transformation and Clonal Analyses of Plasmids in Streptocccus sanguis

Three strains of Streptococcus sanguis, WE4, Wicky, and Challis, were transformed with plasmids derived from Streptococcus pyogenes, strain SM 60 (ERL1), S. sanguis, strain G102 beta (pAM beta 1), Streptococcus faecalis, strain DS5 (pAM beta 1), S. faecalis, strain JH2-2 pAM beta 1 (pAM beta 1), Bacillus subtilis, strain BD 431 (pBD15), and Staphylococcus aureus, strain RN 2442 (pE194). All plasmids from these strains contained erythromycin and lincomycin resistant genes. Two clones from each transformed strain were examined for plasmid content by agarose-gel electrophoresis. Plasmids were isolated from them (by a new isolation procedure) and used to transform other WE4, Wicky, or Challis cells. These, too, were examined by agarose-gel electrophoresis. The results of these clonal analyses showed that some plasmids (ERL1) were more or less conserved, whereas others (pE194, pBD15, and pAM beta 1) were markedly changed. pAM beta 1, the plasmid most intensively examined thus far, underwent large modifications independent of the cells from which it was derived and the cells into which it was introduced by transformation. Eighteen molecular weight sizes have been identified, ranging from 4 to 33 medadaltons. Therefore, some changes resulted from deletions whereas others resulted from additions. It was also shown in S. sanguis WE4 and Wicky cells that the low frequency of transformation was due to a low level of pAM beta 1 DNA in the original S. faecalis, strain DS5, preparations. Challis cells were still poorly transformable with the same DNA preparations, indicating a possible host-cell modification system different from the one in WE4 or Wicky cells. (Ranhand)

Administrative, Organization, and Other Changes

The Laboratory has been in a state of dissolution, diminishment, and demoralization since indications two years ago that it would probably cease to exist on mandatory retirement (November 1, 1981) of the Laboratory Chief. Without replacement, personnel have left, and continue to leave, for other opportunities. Mr. William O. Mitchell (Biologist) left, on detail through the Intergovernmental Personnel Act on September 15, 1980, for experience in grant procedures and administration at Georgetown University Medical School. Dr. Daniel J. Moynet's appointment as Visiting Associate from France was completed on February 27, 1981, and he took a position at the Universite de Sherbrooke, Quebec. Mrs. Catherine Dixon (Biol. Lab. Tech. Micro.) was reassigned on March 19 to the Office of the Scientific Director, NIAID; and Ms. Blanche Lewis (Biol. Lab. Tech. Micro.) was similarly reassigned on June 28. Dr. Gary Calandra (Medical Officer) resigned from the U. S. Public Health Service and left LSD and NIH on June 29 to take a position with Merck, Sharpe, and Dohme. Mrs. Alba E. Colon-Whitt has applied for disability retirement. Official notice of the disestablishment of LSD, effective May 28, 1981, was distributed in mid-June. It is expected that remaining personnel will be reassigned within the Institute--the majority, if not all, to the newly-established Laboratory of Molecular Microbiology--but personnel actions have not been completed at the time of this writing. The cumulative loss of one-third of the Laboratory staff, accompanied for most of the year by lack of definitive information as to career futures, has resulted in a decrease in the extent and vigor of research activities. This is the final report of this Laboratory, which began as the Laboratory of Microbiology 13 years ago. All projects are terminated with this report.

Honors and Awards

R. M. Cole

Member, Editorial Board, *Infection and Immunity*

Member, Study Group on Viruses of Mycoplasmas and Spiroplasmas: Subcommittee on Bacteriophages, International Committee on Taxonomy of Viruses.

President, Lancefield (Streptococcal) Society, 1980-81.

E. Huff

Invited participant, United States-Australia Cooperative Science Program Joint Workshop entitled, "Chemistry and Biological Activities of Bacterial Surface Amphiphiles", January 12-18, 1981.

J. M. Ranhand

Presented paper at the 1980 Mid-Atlantic Extrachromosomal Element Group, Sept. 26-28, 1980, Charlottesville, Va.

Presented seminar at Temple University Medical College, Jan. 5, 1981.

Presented paper at the 25th Wind River Conference on Genetic Exchange, June 8-12, 1981, Estes Park, Colorado.

T. S. Theodore

Invited participant, United States-Australia Cooperative Science Program Joint Workshop entitled, "Chemistry and Biological Activities of Bacterial Surface Amphiphiles", January 12-18, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00001-20 LSD

PERIOD COVERED

October 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical Studies on Staphylococcal and Streptococcal Membranes, Cell Walls, and Mesosomes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. Huff	Sci. Director	LSD, NIAID
Other:	T. S. Theodore	Res. Microbiol.	LSD, NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lipoteichoic acid, a major amphiphilic molecule of Gram positive bacteria, occurs in membranous organelles known as mesosomes. The purpose of this work has been to determine the functional role of lipoteichoic acid (LTA) and mesosomes in the growth of the staphylococcal and streptococcal cells. Present studies have been directed at determination of some of the unique properties of LTA, namely, the density, degree of aggregation, and affinity to various surfaces. We have demonstrated that LTA exists in several different interconvertable aggregation states. Recently, we separated staphylococcal mesosomes into two distinct density types which were further fractionated by osmotic lysis and centrifugation.

7

Project Description

Intracellular membranous organelles known as mesosomes, in addition to plasma membranes, occur as characteristic morphological features of Gram positive bacteria. *Staphylococcus aureus* mesosomes can be readily prepared free of plasma membranes. Such mesosomes contain 18% lipoteichoic acid (LTA). The presence of LTA is the major chemical feature which distinguishes mesosomes from plasma membranes. Although lipoteichoic acids have been known since their discovery 10 years ago to contain both a hydrophobic and hydrophilic center, there has been no study of their unique amphiphilic properties. The formation of micellar aggregates of LTA which are excluded from 6% agarose gels in aqueous solution is an essential part of most LTA purification procedures. However, the rates of association and dissociation, critical micellar concentration (CMC), and micellar size of these aggregates is unknown. Admixture of LTA with Triton X-100 is necessary for the expression of the biological properties of LTA and for LTA recovery from DEAE Sephacel. This suggests the importance of micellar size for the expression of biological activity. As a basis for further studies on the biological properties of LTA and to understand the source of LTA losses during purification, studies have been directed at the effect of time and LTA concentration on (1) the sizes and amounts of LTA micelles and, (2) the tendency of LTA to absorb to surfaces. We have separated mesosomes from *S. aureus* (Lafferty) into two density types, heavy and light, and have initiated a study to determine possible chemical, topological, and physiological differences in the two mesosomal types.

1. Amphiphilic Properties of Lipoteichoic Acid

LTA is routinely separated into a high and a low molecular weight fraction (LTA-I and LTA-II) on Sepharose 6B. We have used sucrose gradient (5-20%) centrifugation to further examine the size distribution of LTA-I and LTA-II and also to measure dissociation of LTA aggregates. After two passages through Sepharose 6B, and concentration by ultrafiltration, LTA-I had a density of 1.3, centrifuged on a sucrose gradient with a Svedberg constant of 8S, and yielded a symmetrical elution pattern on Sepharose 6B. If such purified LTA was dialyzed or allowed to stand at concentrations of 1 ug/ml or less, it dissociated into particles which sedimented at 2S. If the LTA-I was concentrated or allowed to freeze in concentrated solution (3 ug/ml or greater), larger aggregates (11S and 15S) in addition to 8S formed. The 2S particles separated from the top of the gradients and, thus, appeared to be different than LTA-I monomer which is observed (as counts) at the top of the gradient. If the molecular weight of LTA is assumed to be 4000, the 2S aggregates must be 3-5 subunits in size, and the 8S aggregates 30-50 subunits in size (120,000-200,000 NW). Association of LTA monomers may be rapid as compared to dissociation if they behave like the amphiphilic brain gangliosides having very low CMC values. Because of such rapid association and the difficulty in separating monomer LTA from 2S LTA, it has not yet been possible to determine the CMC for the formation of 2S aggregates.

If LTA-I is treated to remove D-alanine, and the D-Ala-LTA peak II isolated on Sepharose 6B, the 2S aggregates so obtained reassociate to 8S aggregates allowing a CMC of 0.8 ug/ml to be calculated for the 2S and 8S association. The CMC values for the association of monomer into 2S aggregates appear to be much lower. This unusually strong tendency to aggregate (low CMC) may: (1) prevent LTA from diffusing out of the cell (as observed when stationary cells are extracted with aqueous phenol), (2) may account for the observed formation of vesicles and membranous tubules which are identified morphologically as mesosomes, and (3) may play an important role in septum formation and cell division in Gram positive bacteria.

2. Recovery of LTA

A comparison of the sucrose gradient patterns of LTA's with the patterns on Sepharose 6B showed that the 8S and higher aggregates of LTA observed on sucrose gradients eluted from Sepharose 6B as peak I and that 2S aggregates eluted as peak II.

If LTA-I was dialyzed and lyophilized prior to chromatography on Sepharose 6B, recovery was poor (20 to 60%). Recovery was improved (70 to 100%) when samples were run on 5-20% sucrose gradients. When the sucrose gradient runs were compared to Sepharose 6B chromatography, it was observed that the yield of 2S LTA (16 and 26%) on sucrose gradients was not reflected in a comparable recovery of peak II (5% or less) off of Sepharose 6B.

Recovery of dAla-LTA-I and dAla-LTA-II was good from sucrose gradients (93% and 107%, respectively), and poor from Sepharose 6B (60% and 43%, respectively). Again the losses were greatest in peak II material. Recovery of the LTA absorbed to Sepharose 6B could be accomplished, in part, by elution with Triton X-100 and Brij 76. However, removal of residual LTA was very slow.

Absorption of the LTA's occurred onto hydrophobic surfaces saturating at 5 to 10 nanograms/cm^2. All the LTA's appeared to be fully acylated since they absorbed completely to Siliclad coated glass. LTA-I absorbed to acid washed borosilicate glass and cellulose nitrate. Recovery of LTA from Sepharose 6B columns was dependent on the amount of 2S aggregate present.

Apparently, 2S aggregates can penetrate the gel where they become bound. Elution of the bound 2S material is difficult even with the use of strong detergents. The 8S aggregates do not penetrate the Sepharose 6B and can be recovered quantitatively. The use of sucrose gradients (at 1 ug LTA/ml or greater) avoids this problem of loss and allows for separation of the various LTA aggregates.

Two Density Types of Mesosomes

S. aureus (Lafferty strain) mesosomes have been separated (by equilibrium sedimentation in sucrose gradients) into two types, a heavy and light mesosome (density 1.15 and 1.13, respectively) (yield 62% and 38%, respectively). Upon rapid dilution of the mesosomes into distilled water, rupture of vesicles was accomplished since 90% of the ^{3}H-glycerol label was solubilized (100,000 x g for 2 h). Upon slow dilution into distilled water 81% of heavy and 63% of light mesosomes was solubilized. The pellet which remained had a density in sucrose of 1.13 and 1.10, respectively. Whether the difference between heavy and light mesosomes is due to LTA, protein, or lipid is unknown and currently under investigation. It is possible that the two vesicle types represent normal and inside-out vesicles.

Planned and onging work includes: (1) Identification of specific in vivo LTA-protein (and LTA-lipid) interactions by 2-dimensional gel electrophoresis of proteins from plasma membranes, heavy and light mesosomes, and fractions of osmotically lysed mesosomes; (2) Determination of the LTA content, degree of fatty acid acylation, and D-alanine ester substitution on LTA in heavy and light mesosomes; and (3) Measurement of the effect of acylation and D-alanine ester substitution on LTA affinity to surfaces and on micellar dissociation.

Publications

Huff, E.: Aggregation of Staphylococcal lipoteichoic acid. In "Chemistry and Biological Activities of Bacterial Surface Amphiphiles" (eds., G. D. Shockman and A. J. Wicken). Academic Press, New York. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00002-16 LSD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the nutrition, physiology and ultrastructure of Staphylococcus aureus.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. S. Theodore	Res. Microbiol.	LSD, NIAID
Other:	C. B. Calandra	Med. Officer	LSD, NIAID
	J. E. Ciardi	Res. Biochem.	NCP, NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 2.6	PROFESSIONAL: 1.0	OTHER: 1.6

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The most distinct feature of Gram positive bacteria are the presence of lipoteichoic acid and a membrane system consisting of a plasma membrane and mesosomes. The purpose of this project is to relate membrane structure with function using Staphylococcus aureus as the model test system. We have already shown by chemical analysis and serological methods on isolated fractions that the majority of the lipoteichoic acid is localized in the mesosomal portion of the membrane. This past year we have investigated some of the functions ascribed to lipoteichoic acid. Using purified preparations of lipoteichoic acid and derivatives prepared from S. aureus, and antisera prepared to the polyglycerolphosphate backbone portion of the lipoteichoic acid, we obtained evidence that this polymer may act as a protein carrier: more specifically, it carries streptolysin S, and it may also play a vital role in the adherence of bacteria to tooth surfaces.

PHS-6040
(Rev. 10-76)

Project Description

Morphologically, the most distinct feature of Gram positive bacteria are the mesosomes which arise from the membranous septum and precede cross-wall formation. The most distinctive feature of isolated mesosomes is the presence therein of almost all of the cell's lipoteichoic acid (LTA, a glycerol phosphate polymer containing a phosphorylated glycolipid). LTA interacts strongly with divalent cations, and acts as a regulator of autolytic activity. Also it is immunologically active, binds to erythrocytes and mammalian cells, may act as a carrier of proteins, and may be involved in the adherence of bacteria to tooth surfaces. Using the LTA isolated from mesosomal vesicles of S. aureus and antisera prepared to backbone LTA (polyglycerol phosphate), we investigated the role of LTA as a carrier of proteins (streptolysin S) and its presence in dental plaque and in involvement in dental caries.

1. Activation of Streptolysin S by LTA

Group A streptococci produce an oxygen stable extracellular toxin (SLS) which attacks not only erythrocytes, but also other mammalian cells. To be active, it requires a carrier molecule such as yeast RNA-core or non-ionic detergents; neither of these is a native component of streptococci or mammalian cells. Intracellularly, the SLS precursor is tightly bound to the membrane and can be activated only by sonication with a carrier or by blending on a vortex mixer with glass beads and a carrier. Because of its cell membrane binding affinity and its possible role in the pathogenesis of streptococcal infections, we tested whether LTA could act as a carrier and activate SLS. When LTA extracted from whole cells of S. aureus by hot phenol treatment and purified by gel chromatography on Sepharose 6b was added to streptococcal membranes, hemolysin was produced and was equal in activity to or better than that produced with the same amount of yeast RNA-core. LTA extracted from the same or different strains of Group A streptococci also activated precursor SLS, as did the LTAs prepared from groups B, C, and G streptococci. Cold aqueous phenol-extracted LTA was by far the most efficient carrier, in comparison with LTA extracted by other methods. When deacylated LTA (either chemically extracted or isolated from the periplasm following protoplasting) and ribitol teichoic acid was tested as carrier, SLS precursor activity was negligible. Also, no inhibition of SLS activity occurred when increasing concentrations of deacylated LTA were added to an activation mixture containing the fully acylated LTA. This suggests that the fatty acid portion of the molecule is involved in the binding of the hemolytic moiety and parallels other studies showing that LTA with an intact glycolipid moiety is necessary for biological activity. Added proof that the hemolytic moiety is bound to LTA was shown by the inhibition of SLS activity when LTA was treated with LTA antisera either prior to or during the activation step. Although membrane bound LTA was released into the supernatant fluid during the activation step, no SLS activity could be detected without an added carrier. This released LTA was deacylated, as ascertained by its inability to sensitize erythrocytes and its elution profile on Sepharose 6b. Similar results were obtained when we tested for the production of extracellular SLS using resting cells prepared from a late log phase culture of group A streptococci. Streptococcal LTA was equal to or better than that obtained with a similar amount of yeast RNA-core. Polyacrylamide gel analysis and gel chromatography on Sepharose 6b of the LTA-SLS complex showed that the hemolysin migrated with the LTA. Also, we showed that the hemolytic moiety could be readily transferred from one carrier to another without much loss in activity. Our results demonstrate that a native component of streptococcal membranes; LTA may function as a physiological carrier of SLS. LTA resembles RNA-core in having a negatively charged repeating polymer backbone, and it resembles nonionic detergents in being micellar. LTA could serve as a carrier of SLS, and possibly as a carrier of other proteins, and deliver it to host sites distant from the site of production or as a surface component mediate bacteria to host cell interaction. (In collaboration with G. B. Calandra)

2. Extraction and Detection of LTA in Dental Plaque

Bacteria which initially colonize the tooth surfaces are Gram positive cocci which apparently possess strongly negatively charged phosphate polymers (LTA) on their surfaces. It has been postulated that LTA possesses physicochemical groups responsible for the adhesion of bacteria to teeth _in vivo_. _Streptococcus mutans_ and _Streptococcus sanguis_ produce significant amounts of extracellular LTA and it has been proposed that the binding of these bacteria to teeth in the presence of dietary sucrose may be mediated by complexes of LTA and insoluble polysaccharides in the bacterial coat. These polysaccharides contain bound phosphate and are negatively charged and capable of binding to calcium, and thus may have a strong affinity for tooth surfaces. For this study we examined plaque samples taken from irradiated monkeys maintained on a high sucrose diet. A 50 ug plaque sample was separated by centrifugation into plaque solid (35 ug) and plaque fluid (25 ul) samples. Each sample was extracted with chloroform/ methanol (1:2), treated with 80% hot aqueous phenol, and analyzed for LTA by passive hemagglutination inhibition using antisera prepared to highly purified LTA (polyglycerol phosphate backbone). The plaque solid material contained most of the LTA and gave a titer of 1:256, while a titer of only 1:16 was obtained with the plaque fluid. The results were consistent and repeatable and would allow for the detection of small amounts of LTA in individual plaque samples. Attempts are now underway for the quantitation of LTA in plaque samples using rocket immunoelectrophoresis rather than the hemagglutination inhibition method which is only semi-quantitative. Possibly a vaccine against dental caries may be developed. Antibody against the LTA polysaccharide complex might prevent or reduce colonization of the tooth surface by the bacteria or affect the diffusion properties of plaque and thus reduce cariogenicity. (In collaboration with J. Ciardi, NCP, NIDR)

Honors and Awards

Invited participant, United States-Australia Cooperative Science Program Joint Workshop entitled, "Chemistry and Biological Activities of Bacterial Surface Amphiphiles", January 12-18, 1981.

Publications

Theodore, T. S., and Calandra, G. B.: Streptolysin S activation by lipoteichoic acid. Infect. Immun. (In press)

Theodore, T. S., and Calandra, G. B.: Streptolysin S carrier activity of lipoteichoic acid. In "Chemistry and Biological Activities of Bacterial Surface Amphiphiles" (eds., G. D. Shockman and A. J. Wicken) Academic Press, New York. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00004-21 LSD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Electron Microscopy of Bacteria in Relating Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. M. Cole	Chief	LSD, NIAID
Other:	T. J. Popkin	Chemist	LSD, NIAID

COOPERATING UNITS (if any)

See following page for cooperating units.

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project provides support, through preparative techniques (negative staining, thin sectioning) and subsequent examination, for Laboratory and collaborative microbiological studies requiring transmission electron microscopy. Objects of ultrastructural study include mycoplasmas and their viruses; streptococci and their bacteriophages; staphylococci; bacilli; gram-negative bacteria; fungi; and various cell fractions thereof, examined by means of cytochemistry and immunoelectron microscopy.

Cooperating Units

Laboratory of Clinical Investigations, NIAID, Clinical Immunology Section (M. Berger, E. J. Brown, S. W. Hosea, K. Joiner)

Laboratory of Clinical Investigations, NIAID, Clinical Mycology Section (K. J. Kwon-Chung)

Laboratory of Infectious Diseases, NIAID, Mycoplasma Section (J. G. Tully)

Dept. of Pediatrics, Infectious Disease/Pulmonary Division, School of Medicine, The University of North Carolina at Chapel Hill (W. A. Clyde, Jr., A. M. Collier, P. C. Hu [EPA])

Dept. of Microbiology, The University of Texas Health Science Center at San Antonio (J. B. Baseman)

Medical Research Council Clinical Research Centre, Harrow, Middlesex, England (D. Taylor-Robinson)

Divison of Bacterial Products, Bureau of Biologics, FDA (M. C. Hardegree, W. H. Habig), and Dept. of Membranes and Ultrastructure, The Hebrew University-Hadassah Medical School, Jerusalem (S. Rottem)

Agricultural Research Service, U.S.D.A., Beltsville, Md. (D. Stiller, R. F. Whitcomb)

Project Decription

Objectives are to provide, as required, electron microscopy facilities in support of other projects in the Laboratory; and to conduct such original and in depth studies of the ultrastructure of microorganisms as may be engendered by collaborative beginnings or by independent approach to problems.

1. Collaborative studies (with S. Rottem, M. C. Hardegree, W. H. Habig: Hadassah and FDA) showed that the clostridial hemolysin and toxin, tetanolysin, in purified form, could be visualized by electron microscopy of negatively stained preparations as arcs and rings of 25-30 nm internal diameter. It thus resembles cereolysin and perfringolysin, and this evidence further dispels the previous concept that these oxygen-labile hemolysins can form arcs and rings only as a result of interaction with cholesterol. In mixtures with liposomes of different cholesterol content, diluted tetanolysin bound to and reacted with liposomes in order of increasing cholesterol content or cholesterol-lipid ratio and electron microscopy confirmed the appearance of arcs and rings, leading to liposome fragmentation, under these conditions. It appears that toxin subunits, because of an affinity for cholesterol, concentrate

locally on or around the liposomes and the concentration leads to polymerization into visible arcs and rings: such are formed similarly in highly concentrated and purified preparations without cholesterol.

2. Electron microscopy of a Moroccan strain of a helical mycoplasma (spiroplasma: strain B63) confirmed a previous report (Ricard, Garnier, Bove, Abstr. 3d Conf. Internat. Org. Mycoplasmology, Sept. 39, 1980, Custer, S. D.: Abstr. #91) of the occurrence of a new and fourth spiroplasma virus. The small (22-25 nm) isometric virus was found to be propagable on U. S. bee spiroplasma isolate BC3. Further studies were precluded by loss of technical personnel from the Laboratory.

3. A new mycoplasma, isolated from the human male urogenital tract by D. Taylor-Robinson (Harrow) and J. G. Tully (LID, NIAID) and tested culturally, serologically, and biochemically by them, was examined by electron microscopy as part of its characterization. The organism, serologically distinct from all known mycoplasmas, is predominantly flask-shaped in morphology, contains a rod-like terminal structure and cap in the narrow tip, and the anterior two-thirds of the flask is covered by a heavy, often particulate, outer layer or nap by which it adheres to red blood cells or cells in cell culture. The specialized ultrastructural features resemble (but appear more advanced than) those of other flask-shaped mycoplasmas such as *M. pneumoniae* (human respiratory tract), *M. gallisepticum* (avian respiratory tract), *M. alvi* (bovine urogenital and gastrointestinal tracts) and *M. sualvi* (porcine urogenital and gastrointestinal tracts), and comparative studies are in progress. A preliminary report (see Publications) has been published and a definitive taxonomic description is in preparation. Although isolated from 3 cases of non-gonococcal urethritis, and only by use of a new culture medium, the new mycoplasma cannot be firmly confirmed as a cause of disease until more extensive clinical and animal studies, now under way, are completed.

4. During comparative studies of the ultrastructure of flask-shaped mycoplasmas with terminal structures, a well-defined particulate nap--similar to, but less extensive than that of the new human genital mycoplasma--was resolved by electron microscopy of negatively-stained wild-type strains of *Mycoplasma pneumoniae*. Such strains are hemadsorbing and cytadsorbing and adhere by the cell tip area covered by the nap. An outer glycoprotein layer in this area was previously postulated on the basis of cytochemical staining of sections by ruthenium red and tannic acid and presumed to be functional in hemadsorption, but no layer was previously seen on untreated specimens. Since hemadsorption-negative strains, occurring either naturally in high passages (Lipman, Clyde, Denny; J. Bacteriol. 100, 1037, 1969) or as a result of chemical mutagenization (Hansen, Wilson, Clyde, Baseman; Infect. Immun. 32, 127, 1981) have been reported, it was of interest to

examine such strains. These were obtained (from Clyde, North Carolina; Baseman, Texas) and preliminary results show degrees of ultrastructural anomalies and absence of terminal organelles and outer nap in hemadsorption-negative mutants. Continuing collaborative investigation with the strain suppliers is focussing on the relation of the nap to a protein (P1) found to be absent in trypsinized M. pneumoniae cells that no longer adsorb to cells (Hu, Collier, Baseman; J. Exper. Med. 145, 1328, 1977) and will include studies of P1 presence and localization in both wild-type and mutant strains.

5. The C3b complex of complement can be coupled to biotin, which, after C3 is bound in the presence of appropriate antibody, remains available for detection by radioactive label, anti-biotin globulin, or binding to avidin. The latter has been conjugated to ferritin by workers in LCI to supply an electron-dense label, and is being applied to attempts at precise localization and enumeration of C3 binding sites under different conditions, on erythrocytes (Berger, Hosea), pneumococci (Brown), and Salmonella (Joiner). Preliminary results of our electron microscopic portion of the collaboration demonstrate the feasibility of detection and photography of the ferritin label by direct examination of otherwise untreated red cell ghosts and bacteria; but definitive findings in this collaboration of only two months duration must await completion of ongoing study.

6. Preparation and electron microscopy of protein film spreads of DNAs of streptococcal bacteriophages were repeated and completed. (See Projects No. Z01 AI 00006-10 LSD and Z01 AI 00005-16 LSD)

7. Several fungi were examined by electron microscopy, in collaboration with the Clinical Mycology Section, LCI (K. J. Kwon-Chung). The basidiomycetous nature, and therefore the classification, of Cryptococcus laurentii was confirmed by the ultrastructural demonstration of characteristic dolipore septa (See Publications). Examination of Exophila jeanselmi, isolated from the only patient on record with widely disseminated disease due to this organism, revealed its ultrastructural details and indicated that unique crystalline formations found in aging cultures are due to accumulation of intracytoplasmic inclusions. A clinical isolate of Alternaria sp., ordinarily a common morphologically stable fungus from air and soil, was found to exhibit unstable morphology; but electron microscopy failed to reveal the presence of intracellular virus-like inclusions suggested by other investigators as a cause of the instability.

8. Sections of control and Eperythrozoon wenyoni-infected calf bloods (Stiller and Whitcomb, U.S.D.A.: received in supplementation of prior samples) confirmed the procaryotic mycoplasma-like ultrastructure of the erythrocyte-associated microorganism.

9. During the year, numerous examinations of negatively-stained mycoplasmas were conducted to affirm morphologies and ultrastructure as an aid to taxonomy. Included were new spiroplasma isolates (Y32, Y38) from Ixodid ticks, cloned strains of a non-helical spiroplasma mutant (ASP-1), an unidentified mycoplasma (SLH-9) from Lepidoptera, avian strains of Mycoplasma synoviae, and several strains of Mycoplasma pulmonis. An ongoing comparative study of flask-shaped mycoplasmas with terminal organelles involved negative stains, ruthenium red staining, and conventional sections of cultures (and infected cell cultures) of 7 strains (including mutants) of M. pneumoniae, and strains of M. pulmonis, M. alvi, M. sualvi, M. gallisepticum, M. 70-159 (Serogroup 38), and a new human genital isolate. It was found that the size (degree of development) and ultrastructure of the terminal organelles, and the extent and nature of outer layers or naps, differed from species to species; and that most, but not all, species appeared to hemadsorb or cytadsorb by mediation of the outer layer. M. 70-159 (Serogroup 38), not previously investigated morphologically, and the new genital isolates G37 and M30, were found to be new members of this group of morphologically similar mycoplasmas.

Publications

Calandra, G. B., and Cole, R. M.: Lysis and protoplast formation of Group B streptococci by mutanolysin. Infect. Immun. 28: 1033-1037, 1980.

Cole, R. M., and Popkin, T. J.: Electron microscopy. In Manual of Methods for General Bacteriology (P. Gerhardt, ed.). American Society for Microbiology, Washington, D. C., Chap. 4, pp. 34-51, 1981.

Tully, J. G., Taylor-Robinson, D., Cole, R. M., and Rose, D. L.: A new mycoplasma from the human genital tract. Lancet 1 (June 13): 1288-1291, 1981.

Cole, R. M.: Transmission electron microscopy: basic techniques. In Manual of Methods in Mycoplasmology, Vol. I (S. Razin and J. G. Tully, eds.). Academic Press, New York. (in press)

Cole, R. M.: Virus detection by electron microscopy. In Manual of Methods in Mycoplasmology, Vol. II (J. G. Tully and S. Razin, eds.). Academic Press, New York. (in press)

Cole, R. M.: Spiroplasma viruses. In ibid. (in press)

Rhodes, J. C., Kwon-Chung, K. J., and Popkin, T. J.: Ultrastructure of septal complex in hyphae of Cryptococcus laurentii. J. Bacteriol. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00005-16 LSD

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on bacteriophages and genetics of streptococci.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Colon-Whitt	Microbiol.	LSD, NIAID
	R. M. Cole	Chief	LSD, NIAID
Other:	D. Moynet	Vist. Assoc.	LSD, NIAID

COOPERATING UNITS (if any)

Laboratory of Parasitic Diseases, NIAID (T. Mercado)

LAB/BRANCH

Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	1.2	1.4

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project has been to elucidate the nature of bacteriophages and their role in the mechanisms of genetic exchange (lysogenic conversion, transduction, transformation) and their effect in the pathogenicity and immunogenicity of Group A streptococci. In studies on transduction of the M-protein, a donor strain rich in M-protein was cloned after bactericidal and mouse passages. Further work on erythrogenic toxin strengthens the hypothesis that lysogeny is not essential for the production of ET-B$^+$ toxin. A "cured" ET-A$^+$, B$^+$ strain lost the ability to synthesize ET-A$^+$ but not ET-B$^+$. Four streptococcal bacteriophages isolated from different serological streptococcal groups showed identical lytic patterns, immunological reactions and transduction patterns. In other collaborative studies we showed that ATFII, the extracellular factor from Pseudomonas fluorescens, controlled T. cruzi infection in mice. After extended treatment the parasites disappeared from the blood stream and normal tissue architecture was restored.

(1) Transduction of antigenic markers in Group A streptococci

Transduction of antigenic markers has been impeded because of difficulties in selecting antigenic transductants. In studies preliminary to attempted transduction of M-protein (the cell surface, type-specific, virulence-associated antigen of Group A streptococci), a new model was tested for selection and scoring of transductants positive for a given M serotype. Plastic petri dishes were coated with the immunoglobulin-binding protein A of staphylococci. This was then exposed to antibody specific for the M-protein of the potential donor streptococcus. The surface was then exposed to a mixture of the donor and another serotype; the former, homologous to the plate-bound type-specific antibody, was also streptomycin-resistant. After an adequate time of exposure, followed by washing to remove any unbound streptococci, plate surfaces were overlaid by agar medium with or without streptomycin, the plates incubated, and colonies counted. Control plates utilized normal rabbit serum. Control suspensions were singlemembered suspensions of each of the two serotypes in the mixture, and the cell concentration of each was the same as that of each in the mixture. The theory assumes that only streptococci of the homologous serotype (which would be the desired transductants in an actual transduction experiment) will adhere to the type-specific antibody and thus develop as countable colonies. The tests showed that normal rabbit serum was ineffective and that plates with type-specific antibody selected from 60-70% of the input number of homologous streptococcal colony-forming units from the mixture. Data from control suspensions showed that a small background of the heterologous streptomycin-sensitive strain adhered non-specifically to both antibody- and normal serum-coated plates, but was eliminated from scoring on plates containing streptomycin. Thus, this model of selection by type-specific antibody and streptomycin appears feasible for adaptation to experiments on transduction of M-protein. However, further technical refinements are needed, and studies have been discontinued becasue of disestablishment of the Laboratory.

(2) Erythrogenic toxin

Erythrogenic toxins (ET or streptococcal pyrogenic exotoxin, SPE) are extracellular proteins produced by a number of Group A streptococci. Four distinctive antigenic types (A, B, C, and D) have been described and lysogeny has been considered essential for their production. However, prior results obtained in this laboratory and confirmed elsewhere, suggest that the genetic coding for ET-B is not on a prophage genome that transcribes a lysogenic conversion to toxin production. This hypothesis is based upon the following findings: (1) a rare non-lysogenic strain was found that produces only ET-B and, upon lysogenization with a phage from an ET-C$^+$ strain, also synthesizes ET-C$^+$, (2) ET-B$^-$ strains failed to become ET-B$^+$ when lysogenized with phages from several ET-B$^+$ strains, (3) an ET-A$^+$B$^+$ strain, when "cured" of its prophage, stopped synthesizing ET-A$^+$, but not ET-B$^+$. We were attempting to "cure" five more ET-B$^+$ strains when the work was discontinued.

(3) Streptococcal bacteriophages

In collaboration with Dr. Daniel Moynet, we investigated some biological and physico-chemical characteristics of virulent and temperate phages of streptococci. Four phages, a virulent (øA25) and a temperate (øAT298) phage from Group A and temperate phages from Group G (øGT234) and Group E (øET42), showed DNA similarities as well as terminal redundancy and circular permutation of their genomes. They also have an identical patterns of infections, lysing 12/22 Group A, 2/2 Group C, 2/4 Group G and 0/1 Group E strains. All were neutralized by specific øA25 antisera (1:1000 dilution) and all transduced the Sm marker to the Group A but not to the Group G and E parent lysogenic strains. (See also Project No. Z01 AI 00006-10)

(4) Lysis of *Trypanosoma cruzi* by *Pseudomonas fluorescens*.

In collaboration with Dr. Teresa Mercado (LPD) we have been studying an extracellular factor from *Pseudomonas fluorescens* that exhibits a lytic effect on *T. cruzi* (Tulahuen strain). As previously reported two fractions, ATFI (MW over 20,000) and ATFII (MW approximately 1,000) were obtained by gel filtration chromatography. ATFII, the most active, is resistant to heat (boiling 5 min) freezing (-20 C), and trypsin (25 ug/ml), but is sensitive to pronase (20 ug/ml). *In vivo* studies have indicated that mice injected with a total of 6 gm ATFII over a period of 58 days after IP inoculation of 1,000 trypamastigotes did not develop the typical Tulahuen infection: the highest parasite count observed was 30 flagellates per 10 microscopic fields. Controls injected with buffer instead of ATFII, as well as untreated infected controls, died 13 to 21 days after inoculation with a parasitemia of 25-50 flagellates per single microscopic field. Histological studies of tissues obtained from treated mice at different times after infection indicated that some parasites were able to penetrate and multiply in the liver and spleen, up to 13 days after inoculation, but the usual progression of the infection (in untreated mice) was controlled, the parasite disappeared from the blood, and normal tissue architecture was restored. These results suggested that: (1) the parasites were altered by the factor with consequent interruption of tissue penetration, or (2) they were lysed in the blood stream, reducing the numbers available for penetration, or (3) the tissue stages of the parasites were altered, causing interruption of the amastigote-trypamastigote cycle. At present we are attempting to develop antibodies in rabbits against ATFII. Further studies are planned to clarify ATFII-parasite interactions both *in vitro* and *in vivo*.

Pubications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00006-10 LSD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Competence Development and Genetic Exchange Mechanisms Among Streptococci.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. M. Ranhand	Sr. Scientist	LSD, NIAID
Other:	D. J. Moynet	Visit. Assoc.	LSD, NIAID

COOPERATING UNITS (if any)
Macromolecular Biology Section, LBV (F. Defilippes)
Z01 AI 00126-08

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

I continued studies on plasmid transfer and expression in Streptococcus sanguis cells. I did a clonal analysis of transformed cells to determine the fate of the newly acquired plasmid DNAs. Certain plasmids appeared to enter cells with little or no modifications. These were derived from Streptococcus pyogenes, strain SM60 containing the ERL1 plasmid. Others were changed physically, for example, pAM beta 1 derived from either Streptococcus faecalis, strains DS5 or JH2-2 pAM beta 1, or from S. sanguis, strain G102 beta. The changes observed were deletions in, or additions to, the initial molecular sizes. For example, transformed cultures contained a minimum of 18 molecular weight classes that covered the range from about 4×10^6 to 33×10^6. The events that lead to these changes are not understood.

Project Description

Part I

Last year I examined the transformation efficiency of a number of plasmids isolated from various strains of bacteria. The plasmids contained erythromycin and lincomycin (MLS) resistant genes. I continued this study by examining clones of cells that were transformed with the parent plasmids and clones of cells transformed with the newly acquired plasmids. For example, S. sanguis WE4 cells were transformed with a plasmid (designated pBD15) derived from Bacillus subtilis cells. The transformants were designated WE4 pBD15-1, -2, etc. These clones were then used as a plasmid source to transform other WE4 cells. These new clones were designated WE4 (WE4 pBD15-1) :1, :2, etc. This was done for the three transformable S. sanguis strains, namely, WE4, Wicky, and Challis. Two clones from each transformed population were examined for their plasmid content. These were then compared to the original plasmid and to each other. Plasmids were analyzed by electrophoresis on ethidium bromide containing agarose gels.

The results obtained were as follows. The plasmid designated ERL1 (gm-molecular weight of 24×10^6; derived from S. pyogenes, strain SM 60; obtained from H. Malke), when introduced into S. sanguis strains WE4, Wicky, or Challis, underwent minimal molecular weight changes as follows. When ERL1 was reisolated from WE4 ERL1 its gm-molecular weight was 20×10^6; when reisolated from Wicky ERL1 its gm-molecular weight was 22×10^6; when reisolated from Challis ERL1 its gm-molecular weight was 25×10^6. All randomly picked clones from the above were identical.

When plasmids from these transformants were introduced into WE4 cells the gm-molecular weights changed as follows. WE4 (WE4ERL1) had a gm-molecular weight of 27×10^6; WE4 (Wicky ERL1) had a gm-molecular weight of 26×10^6; WE4 (Challis ERL1) had a gm-molecular weight of 26×10^6. Similar results were obtained when Wicky or Challis cells were used as recipients in the transformation reaction.

The plasmid designated pBD15 (gm-molecular weight of 2.2×10^6; derived from Bacillus subtilis, strain BD431; obtained from D. Dubnau), when introduced into S. sanguis WE4, Wicky, or Challis cells, underwent a more extensive rearrangment than ERL1 above. WE4 pBD15-1 had a gm-molecular weight of 21×10^6; WE4 pBD15-2 had a gm-molecular weight of 4×10^6; Wicky pBD15-1 and -2 had gm-molecular weights of 23×10^6;

Challis pBD15-1 had a gm-molecular weight of 15 x 10^6; Challis pDB15-2 had a gm-molecular weight of 22 x 10^6. The reisolated plasmid underwent molecular plasmids. To do this I modified the procedure of Currier and Nester (Analyt. Biochem. 76: 431-441, 1976). The salient details are now presented for the qualitative identification of plasmids in streptococci (and other Gram positive or Gram negative bacteria). Pellets from 10 ml of overnight cultures of streptococci were treated at pH 12 for 60 min, at 37 C, in 0.05 M Tris-OH. This treatment rendered them more susceptible to the action of lysozyme (2 mg/ml). The cells were placed in a final concentration of 20% w/v sucrose. This was essential for the maintenance of the larger plasmids. After addition of lysozyme, a chelating agent (EDTA) was added. The cells were incubated for 60 min, at 37 C, and lysed with 4% w/v sodium dodecyl sulfate (SDS). After doubling the volume with water, NaCl was added to 0.15 M and ethidium bromide was added to 200 ug/ml. The DNA was sheared through an 18 gauge needle and denatured by heating at 100 C for 15 min and quenching in ice water. After cooling, the mixture was made 3% w/v with NaCl and mixed 10 min at room temperature with an equal volume of phenol. After centrifugation, the plasmid containing fraction was mixed with an equal volume of chloroform-isoamyl alcohol. The plasmid containing layer was precipitated with 2 volumes of ethanol (in the presence of magnesium and phosphate) at -40 C overnight. After centrifugation, the residual ethanol was removed by drying in vacuo. The samples were then dissolved in 0.1 ml, 0.1 M EDTA and 0.01-0.02 ml was put into the wells in a agarose gel. Samples were run in pairs. One from each pair was heated at 100 C for 30 seconds and quenched in ice water. This treatment prior to electrophoresis removed most or all open circular and linear forms of DNA leaving only covalently closed molecules (plasmids).

The salient features of the method for the quantitative isolation of plasmid DNA include the above plus a different denaturation step. Lysates were treated at pH 12.2 to 12.5 overnight at 37 C before being extracted with phenol. This treatment not only denatured DNA but hydrolyzed RNA to dialyzable products. This is essential because RNA can contaminate the plasmid DNA preparation through CsCl-ethidium bromide centrifugation and interfer with the UV determination of the DNA concentration.

This significance of the data obtained with the beta plasmid is that it differs from published reports (a. LeBlanc and Hassell, J. Bacteriol. 128: 347-355, 1976; b. Macrina et al. Plasmid 4: 8-16, 1980). These authors, using only Challis cells, found deletions of two kinds. (1) A 9% deletion (a), and (2) a 60% deletion that is reported to be site-specific (b). My data show multiple site deletions and additions. These results have importance in understanding the mechanism by which cells take up DNA molecules from their environment and what they do with them. This is important since plasmid transfer in streptococci is being considered as a tool for "genetic engineering" type of experiments.

I am planning to continue these studies (in the Laboratory of Molecular Microbiology) with the purpose of defining the mechanisms involved in plasmid uptake, modification, and transformation. This approach falls into the general topic of gene-transfer and expression in streptococci.

Part II

Streptococcal bacteriophages. The previously reported initial characterization of several bacteriophages of streptococci was extended by Dr. Moynet (See also Projects Z01 AI 00005-16 and 00004-21). Six phages were of long-tailed polyhedral morphology and originated from streptococci of Groups A, B, E, G, and H. Of these, one Group A phage (presumably virulent) and 3 temperate phages from Groups A, E, and G, showed a common pattern of lytic infection of 28 indicator strains as well as a close serological relatedness: all had linear double stranded (ds) DNAs of molecular size 23-24 Mdal, that possessed (as shown by 2 different methods) circular permutations and terminal repetitions, and their G+C contents were essentially identical (40.4 to 40.9 mol%). The Group H temperate phage DNA, with a gm-MW of 25×10^6, was also circularly permutated and terminally repetitious, and had a G+C content of 42.3 mol.%. Two virulent phages, one infecting Group C streptococci and one infecting Group H, were morphologically different (short-tailed with accessory fibers). One, Cl, possessed linear ds DNA of gm-MW $10.5\text{-}10.6 \times 10^6$, a G+C content of 37.5 mol.%, and no permutation nor terminal repetition. The other, H39, had a circularly permuted linear ds DNA without terminal repetition, of MW $11\text{-}11.4 \times 10^6$, and with a G+C content of 35.2 mol.%. Further comparisons by restriction endonuclease fragment patterns of DNAs suggest that those of the A, E, and G phages are essentially identical, which raises interesting questions of the origins and distributions of phages among different serogroups of streptococci. The evidence also indicates that short-tailed phages are markedly different biologically and in characteristics of their DNAs, from the predominant long-tailed streptococcal phages. This study was terminated because of the departure of Dr. Moynet, but a report of findings is in preparation for publication.

Hemophilus bacteriophage. The work on physical mapping of the genome of phage N3 of Hemophilus influenzae was completed. (See Publications)

Publications

Ranhand, J. M., Mitchell, W. O., Popkin, T. J., and Cole, R. M.: Covalently-closed circular deoxyribonucleic acids in spiroplasmas. J. Bacteriol. 143: 1194-1199, 1980.

Ranhand, J. M.: Intra- and intergeneric transformation of Streptococcus sanguis cells with MLS resistant plasmids. In G. Mazza and M. Polsinelli (eds.), Transformation--1980, Proceedings of the 5th European Meeting on Bacterial Transformation and Transfection, Florence, Italy, Cotswold Press, Oxford, England. (In Press)

Ranhand, J. M.: Transformation and clonal analyses of three Streptococcus sanguis strains treated with pAM beta 1. In Streips, Wilson, Guild, and Goodgal (eds.), Proceedings of the 25th Wind River Conference on Genetic Exchange, Estes Park, Colorado, M. Dekker. (In Press)

Moynet, D. J., and Garon, C. F.: Streptococcal bacteriophage ∅42 has a terminal repetition and a circular permutation. Virology 109: 211-214, 1981.

Moynet, D. J., and Defilippes, F. M.: Characterization of bacteriophage ∅42 DNA. Virology (In Press)

Filipska, E. S., Moynet, D. J., and Defilippes, F. M.: Characterization of bacteriophage N3 DNA. J. Virol. (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00007-06 LSD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Viruses of Spiroplasmas and Mycoplasmas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. M. Cole	Chief	LSD, NIAID
Other:	T. J. Popkin	Chemist	LSD, NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Owing to loss of key technical personnel, with consequent shrinkage of effort and diversion into new areas of more immediate import, it was not possible to continue prior studies in more than a desultory fashion. Because of disestablishment of the Laboratory (effective May 28, 1981), this project is terminated in its entirety.

PHS-6040
(Rev. 2-81)

Publications

Ranhand, J. M., Mitchell, W. O., Popkin, T. J., and Cole, R. M.: Covalently-closed circular deoxyribonucleic acids in spiroplasmas. J. Bacteriol. 143: 1194-1199, 1980.

Cole, R. M.: Transmission electron microscopy: basic techniques. In Manual of Methods in Mycoplasmology, Vol. I (S. Razin and J. G. Tully, eds.). Academic Press, New York. (In Press)

Cole, R. M.: Virus detection by electron microscopy. In Manual of Methods in Mycoplasmology, Vol. II (J. G. Tully and S. Razin, eds.). Academic Press, New York. (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00181-03 LSD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation, Purification, and Characterization of Toxins and Inflammatory Factors if Streptococci.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. B. Calandra	Med. Officer	LSD, NIAID
Other:	T. J. Popkin	Chemist	LSD, NIAID
	R. M. Cole	Chief	LSD, NIAID

COOPERATING UNITS (if any)

Arthritis and Rheumatology Branch, NIADDK (R. Wilder)

LAB/BRANCH

Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 2.2	PROFESSIONAL: 1.4	OTHER: 0.8

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Streptococci contain numerous toxic substances which may be secreted or be a part of the cellular structure, such as the peptidoglycan as part of the cell wall. Streptolysin S is a very potent hemolysin and membranolytic agent which is formed from an intracellular precursor. An objective is to purify SLS and precursor to determine a potential role in streptococcal disease. The development of arthritis in rats after injection of cell walls (peptidoglycan) is being studied to determine the influence of genetic background on the development of such disease.

PHS-6040
(Rev. 2-81)

Project Description

(1) Streptolysin S

Studies on the purification and activation of SLS precurosr have been extended using the synthetic polynucleotide, polyguanylic uridylic acids (pGU). Earlier apparent differences between the molecular sizes of activated precursor from cytoplasm and that from membrane have been shown to be due to degradation of pGU by endogenous cytoplasmic nucleases. While there now are no major known differences between the two pools of precursor, the effect of RNAse on activated precursor from membranes should prove useful in a scheme to purify SLS. The direction of future study on SLS should be to compare composition of extracellular SLS and that activated from precursor to determine the amino acid composition of SLS.

(2) Peptidoglycan-induced arthritis

When injected intraperitoneally with sonicated streptococcal cell walls, most outbred rats develop polyarthritis. Clinically, the arthritis follows a biphasic course. Acute swelling and erythema of the distal extremities develops within the first week and then subsides. Over the next two to six weeks, some of the injected outbred rats develop a chronic symmetric distal joint polyarthritis of variable severity. To explore the role of host genetic factors in this experimental model, we injected partially purified streptococcal cell walls intraperitoneally into 16 inbred strains of rats. There were marked differences in the various strains in the development of arthritis, unequivocally demonstrating that susceptibility to development of chronic arthritis is dependent upon multiple genes. Further studies have shown that susceptibility to the development of arthritis is a dominant genetic trait. A previously undescribed finding in this model is the presence of noncaseating granulomas in several organs, including liver and spleen, of severely arthritic rats. There is a close correlation between extent of granuloma formation and severity of arthritis; those rats with markedly destructive arthritis had numerous granulomas throughout several different organs, in marked constrast to those with mild arthritis which had few lesions; and to arthritis- resistant rats which had no lesions. Preliminary immunological studies have shown that both susceptible and resistant rats have marked suppression of cell mediated immunity following cell wall injection. Those animals which develop arthritis, however, regain their cell mediated immunity, suggesting that suppression of such responses are protective against the development of disease. Further detailed studies are underway to characterize these processes.

Certain other characteristics that are influenced by genetic background, have also been observed. For example, in a few strains unrelated in their susceptibility to arthritis, injection of cell walls was lethal for a significant number of animals. About 25% of animals from strains RHA or ACI, which did and did not develop arthritis, respectively,died within 72 hrs of injection; but there were no deaths in strains LEWIS or WKY, which did and did not develop arthritis, respectively. Most animals died of uncertain causes but often had hemorrhages in the bowel wall or in the lung parenchyma, or had serosanginous discharge about the eyes, suggesting a bleeding disorder. Further analysis of this phenomenon seems warranted because of its similiarity to rare fulminant hemorrhagic infections with streptococci in humans. (In collaboration with R. Wilder)

Using mutanolysin, a muralysin, we studied the enhancement of identification of streptococci by tests such as Serostat. We were able to identify single colonies of beta-hemolytic streptococci from clinical specimens within five minutes. Further refinement of this technique may make it useful for the clinical laboratory.

Publications

Calandra, G. B., and Cole, R. M.: Lysis and protoplast formation of Group B streptococci by mutanolysin. Infect. Immun. 28: 1033-1037, 1980.

Calandra, G. B.: Effect of detergents on streptolysin S precursor. Infect. Immun. 29: 306-310, 1980.

Calandra, G. B., and Whitt, R. S.: Activation of streptolysin S-related cellular potential hemolysin by grinding with glass beads. J. Clin. Microbiol. 12: 84-87, 1980.

Calandra, G. B., and Mackowiak, P. A.: Retropharyngeal abscess, mediastinitis, and pleural effusion in a patient with streptococcal facial erysipelas and maxillary sinusitis. JAMA (In Press)

Shama, S., and Calandra, G. B.: Atypical erysipelas caused by group G streptococci in a patient with cured Hodgkins disease. Arch. Dermatology (In Press)

Calandra, G. B., and Henson, C. L.: Mutanolysin enhancement of serogrouping of single colonies of streptococci. J. Clin. Microbiol. (In Press)

Theodore, T. S., and Calandra, G. B.: Streptolysin S carrier activity of lipoteichoic acid. In "Chemistry and Biological Activities of Bacterial Surface Amphiphiles" (eds. G. D. Shockman and A. J. Wicken). Academic Press, New York. (In Press)

Theodore, T.S., and Calandra, G. B.: Streptolysin S activation by lipoteichoic acid. Infect. Immun. (In Press)

LABORATORY OF VIRAL DISEASES
1981 Annual Report
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PHS-NIH
SUMMARY REPORT

ANNUAL REPORT OF THE LABORATORY OF VIRAL DISEASES, NIAID

October 1, 1980 to September 30, 1981

Dr. Wallace P. Rowe
Chief, Laboratory of Viral Diseases

The program of the LVD continues to be concentrated on murine C-type viruses and their genetic control. Through collaborative efforts with Dr. Malcolm Martin's group in LMM and Dr. Herbert Morse in LMI, we are conducting a broad program in the biology, biochemistry and immunology of these important agents.

The use of recombinant DNA techniques to provide specific probes for the various types of C-type viruses as well as for subregions of the viral genome has greatly increased our ability to analyse the chromosomal distribution of these agents and should enable us to analyse some of the molecular details of their involvement in carcinogenesis. In conjunction with our large mendelian genetic program and the somatic cell hybrid work these biochemical studies are providing information on the chromosomal location and organization of different genomes with a precision previously unimaginable.

An increasing amount of effort is going into analysis of the viral and nonviral genetic factors that affect the occurrence of various types of hematopoietic neoplasm. Also, the tumors that develop in these studies are being used to examine a number of important questions on the classes of cells involved and on the molecular changes in the endogenous viral sequences.

The collaborative program with LMI investigators has identified a number of unique mouse systems for study of factors regulating the expression of endogenous C-type virus sequences in lymphocytes. Of particular interest are some systems in which virus expression behaves as a recessive phenotype or is affected by genes modifying immune responses. We hope to eventually determine if the marked phenotypic differences in expression of xenotropic virus and antigens in different mice are associated with increased risk of particular hematopoietic neoplasms and to determine if such expression plays a role in regulation of immune responses.

Mapping of MCF virus resistance gene. A mouse gene which specifically controls in vitro and in vivo infection by MCF viruses has been mapped to chromosome 5, very closely linked to the gene for hammer-toe. The close linkage of a highly penetrant morphological gene greatly simplifies the identification of mice segregating for Mcfr in studies of the effect of this gene on viral and chemical carcinogenesis. (Hartley, Morse (LMI), Kozak, Rowe)

Identification of Mcfr in CBA substrains. In addition to DBA/1 and DBA/2, mice of the CBA/H subline (CBA/N, CBA/Ca, CBA/H) have been found to carry the MCF resistance gene Mcfr; mice of the CBA/J subline do not. This finding provides at least a partial explanation of the puzzling observation that CBA/H, a mouse strain expected to be permissive on the basis of Fv-1 and H2 genotype, was in fact suppressive to spontaneous leukemia in crosses with AKR. (Hartley, Rowe)

Mcfr gene suppresses ecotropic virus induced erythroleukemia. Mice segregating for the Mcfr gene from DBA/2 were inoculated as babies with Friend ecotropic virus. The development of erythroblastic leukemia was reduced in frequency and delayed in latent period in those mice which were typed (by challenge of tail tissue cultures) as resistant to MCF virus infection. (Hartley, Rowe)

A biological activity of MCF virus identified in molecular recombinant virus. Preliminary data suggest that one characteristic property of those MCF viruses which are thymotropic and leukemogenic, a high SC-1: NFS tissue culture infectivity ratio, has been preserved in a DNA recombinant constructed from the left hand portion of AKV ecotropic virus viral DNA and a small right hand piece of AKR-247 MCF DNA. (Hopkins (MIT), Hartley)

Locus for xenotropic virus carried on chromosome 1 in many mouse strains. In seven inbred strains a xenotropic virus-inducing locus has been located on chromosome 1. In 6 of these strains the sites are allelic; this locus is designated Bxv-1. In one strain, F/ST, a single locus has also been identified and mapped to chromosome 1 but present data suggest that it may be non-allelic with Bxv-1. (Kozak, Rowe, Morse (LMI), Hartley)

C-type virology of the Asian mouse M. molossinus found to be complex. The Japanese mouse M. molossinus is of particular interest because of the role played by related animals in the origins of laboratory mice. M. molossinus has been found to carry two or more ecotropic virus loci and 3 loci for xenotropic virus. There appears to be multiple gene control of expression of spontaneous ecotropic virus and of resistance to Friend SFFV disease. (Kozak)

Ecotropic virus regularly induces myelogenous leukemia. A strain of ecotropic MuLV dervied from spleen of a normal adult wild mouse has been found to reproducibly induce myelogenous leukemia in inoculated laboratory mice after a latent period of several months. The pathophysiology and virology of this disease are being studied to determine if this represents a valid model system for human chronic myelogenous leukemia. (Fredrickson, Hartley)

B cell lymphomas associated with murine C-type viruses. Spontaneous lymphoid cell neoplasms occur in a high proportion of NFS Swiss mice congenic for various ecotropic V loci derived from AKR and other high leukemia mouse strains. The occurrence of these tumors is correlated with high levels of ecotropic virus early in life, and the majority of tumors tested contain MCF viruses. In contrast to the high frequency of T cell lymphomas characteristic of AKR mice, many of the neoplasms in the congenic mice are of B cell origin. (Fredrickson, Morse (LMI), Hartley).

Heterogenity of ecotropic-related sequences within substrains of AKR mice. A molecularly cloned probe specific for ecotropic MuLV has been used in conjunction with restriction enzymes to study the organization of ecotropic proviral genes in AKR/N, AKR/J and AKR/Boyse mice. Three to nine proviral genes have been found with the pattern for each strain remaining relatively stable. (Buckler, Martin, Rowe)

Biochemical detection of xenotropic proviral DNA. A 500 base pair fragment of a xenotropic MuLV proviral envelope gene has been molecularly cloned and characterized. Its use as a xeno-specific probe in combination with previously described ecotropic specific and general MuLV probes has provided valuable information regarding the organization of endogenous retroviral sequences in various mouse DNAs which is needed for a better understanding of virus expression and leukemogenesis. (Buckler, Hoggan, Martin, Rowe)

Organization of endogenous xeno-specific retroviral sequences in mouse genomic DNA. Restriction endonuclease analyses of genomic DNA from 10 different mouse strains with the Southern blotting technique and a xeno-specific recombinant DNA probe have shown that all strains of mice contain many copies of xeno-specific retroviral DNA. In each mouse strain these endogenous xeno-specific retroviral sequences are organized into the same multiple families of sequences allowing for the definition of a "general" xeno-specific restriction map. (Hoggan, Buckler, Martin, Rowe)

Changes in adeno-associated virus (AAV) genome during long term passage in carrier cells. Minor changes in the terminal portions of AAV genome integrated into human carrier cells resulted in the appearance of a few copies of non-integrated DNA. Late cultures demonstrating these small amounts of "free" AAV DNA are more readily induced to produce AAV than earlier cultures containing only integrated AAV DNA. (Berns, Hoggan)

Acetylation of adeno-associated virus (AAV) polypeptides. All of the three polypeptides recovered from mature AAV virions were found to be acetylated on their n-terminal amino acids. Such acetylation took place in the nucleus and cytoplasm even after new protein synthysis was blocked with inhibitors. (Johnson, Hoggan)

Honors and Awards

Dr. Rowe was one of the recipients of the General Motors Cancer Research Foundation Award for 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00011-16 LVD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Studies of small DNA containing viruses belonging to the family Parvoviridae.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. D. Hoggan, Staff Scientist, LVD, NIAID

COOPERATING UNITS (if any) K.I. Berns, Univ. of Florida, College of Medicine, Gainesville, Florida; F. Brent Johnson, Department of Microbiology, Brigham Young University, Provo, Utah.

LAB/BRANCH
Laboratory of Viral Diseases

SECTION
Viral Oncology Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of the adeno-associated virus (AAV) DNA in different laboratory derived carrier clones has demonstrated some changes in the virus genome after several years in passage as integrated DNA in host cells. Studies of parvovirus polypeptides have shown that they are acetylated and such acetylation occurs even after blocked protein synthesis.

Project Description

Objectives: New molecular biological approaches in concert with classic virological techniques are used to investigate the various interactions of parvoviruses and their host cells. These studies should lead not only to a better understanding of the mechanisms of parvovirus replication and their persistence but also to a deeper understanding of eukaryotic host cell function.

Major Findings

Changes in the adenovirus-associated (AAV) genome during long term culture of AAV carrier cells: Continuing studies of the structure of the AAV genome in carrier cells have shown that minor alterations in the genome itself as well as in its integrated state do occur. Small changes in the terminal positions of some of the DNA of the "carried" AAV DNA result in the presence of a few "free" epi-chromosomal non-integrated copies of virus DNA. Cells carrying these "free" copies of AAV DNA appear more easily induced to express whole AAV virus by superinfection with adenovirus or herpesvirus helper, than are cells carrying only AAV genome integrated into the carrier cell genome.

Acetylation of parvovirus polypeptides: Earlier studies which attempted to define the N-terminyl amino acid composition of various parvovirus polypeptides using the danzylation procedure failed because they appeared to be blocked. We have now found that they are indeed blocked by an acetate group and such acetylation takes place in infected cells both in the cytoplasm and in the nucleus even after protein synthesis is blocked. Whether the acetylation plays a role in virus assembly is currently under study.

Significance to Biomedical Research and the Program of the Institute

Our studies during the last 10-15 years as well as those of others have documented a slow but not necessarily steady evolutionary change in parvoviruses. Such changes can be rapidly accelerated in the laboratory and are indeed responsible for the successful production of attenuated vaccines for such diseases as feline panleukopenia and mink enteritis. Our studies of the AAV genome and of virulence changes we have noted in rat virus offer the possibility of in depth study of such changes at the molecular level. The explosive appearance of a "new" frequently fatal canine parvovirus disease created a near panic among dog owners throughout the world a few years ago. This finding has added impetus for a deeper understanding of the changes that occur in the parvoviruses. This is particularly emphasized when it is realized that the so called "new" canine parvovirus was in fact the old panleukopenia virus of cats with a new host range. Was its appearance the result of limited growth or a few base changes in the cat vaccine strain? Such questions are of course of great importance to the study of vaccine dangers as well as efficacy of such vaccines. The isolation of several parvoviruses from human tissues and fluids without any apparent association with disease still remains a question of interest.

Proposed Course

Actual work on this project is currently taking place at a much reduced rate as work in other areas is still taking precedence. However, the PI is still very interested in these viruses and continues to collaborate with those in the field. This collaboration will continue at this same low level at least for the present time.

M. David Hoggan delivered two seminars during the current fiscal year. The first was at Johns Hopkins University, Department of Pathobiology and the second at the University of Connecticut, Laboratory of Pathobiology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00012-19 LVD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of Mouse Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Dr. Wallace P. Rowe, LVD, NIAID
Dr. Janet W. Hartley, LVD, NIAID
Dr. Christine Kozak, LVD, NIAID
Dr. M. David Hoggan, LVD, NIAID
Dr. Janet M. Moore, LVD, NIAID (3 months)
Dr. Jonathan Silver
Mr. Charles Buckler

COOPERATING UNITS (if any) M. Martin, OSD, NIAID; H. Morse, LMI, NIAID; M. Potter, LCBGY, NCI; L. Old, E. Stockert, Memorial Sloan-Kettering Inst.; M. Collins, MBA; P. Pitha, S. Staal, Johns Hopkins Univ.; N. Hopkins, MIT; M. Cloyd, RML.

LAB/BRANCH
Laboratory of Viral Diseases

SECTION
Viral Oncology Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
14.4	6.0	8.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The natural biology of the complex group of murine C-type RNA viruses is being studied using virological, genetic, and biochemical approaches. Continuing studies of the genetic transmission of both ecotropic and xenotropic virus classes have led to the chromosomal mapping of additional virus-inducing loci. Studies of the recombinant MCF viruses in relation to murine leukemogenesis have delineated certain biological and biochemical correlates of the ability of a given MCF isolate to induce or accelerate leukemia, leading to the recognition of target tissue host range as the major determinant of leukemogenicity. Sensitivity to MCF virus infection has been shown to be under genetic control and the relationship to lymphoid and erythroid neoplasia of MCF virus infection, spread and/or generation is under study. Endogenous virus genomes in various inbred and wild mice are being analyzed using defined biochemical probes derived from molecular cloning of ecotropic, xenotropic and MCF viruses; these include cross-reactive and ecotropic and xenotropic specific viral genome fragments.

Genetic factors in susceptibility to spontaneous viral lymphoma of strain AKR. We have shown previously that AKR lymphomagenesis involves generation of novel recombinant thymotropic MCF viruses. We have initiated a series of genetic crosses between AKR and other inbred strains to identify genes that affect lymphomagenesis at different stages before and after formation of MCF virus. We are determining the sensitivity of the hybrids to spontaneous and MCF-247-induced thymomagenesis in relation to their Fv-1, Mcfr, and H-2 types; of especial interest would be a strain combination that allowed formation and spread of MCF virus without thymomagenesis. Results to date indicate that the Fv-1 type is the most important determinant; Fv-1^b virtually eliminates the disease, and Fv-1^{nr} produces a long delay in onset. The Mcfr resistance allele also delays onset of both the spontaneous and MCF-induced disease. An additional gene for partial resistance is present in C57BR and C57L.

The studies of F_1 hybrids fully confirm the crucial importance of MCF viruses in AKR disease. All systems that delay the disease are associated with absence of thymic MCF virus during the latent period, while all sensitive systems develop MCF viruses; further, almost all of the thymomas that do develop in resistant mice also contain MCF's.

Studies of NFSx(NFSxAKR) backcross mice challenged with MCF-247 virus show that inheritance of endogenous ecotropic virus is essential for leukemogenesis. This effect may involve helper effect and/or suppression of immune response.

A careful virologic-pathologic study is being made of NFS congenic mice carrying ecotropic virus-inducing loci derived from AKR, C3H/Fg, and C58 mice. The incidence and histologic spectrum of spontaneous neoplasms in these mice is not related to the genetic source of the locus, but only to the titer of virus induced in early life. This is consistent with the model that spreading virus is required for tumor induction and that local genetic effects in the region of the endogenous locus are not involved.

Spontaneous neoplasms among these mice have been classified in a histopathologic survey of tissues from mice dying or terminated at the end of an 18 month observation period. This survey covered 1979, 1980 and the first half of 1981. Most commonly observed were lymphomas of both T and B origin with chronic myelogenous leukemia, the other neoplasm of the hematopoietic system, being seen much less frequently. Since all AKR mice die with spontaneous T cell lymphomas by a year of age, and NFS/N mice have a very low incidence of lymphomas, it appears that congenic animals have an intermediate incidence. Thus the leukemogenic effect of the inserted retrovirus genes appears to be modified by the NFS/N background but they remain highly effective in causing neoplasms. There appears to be no effect of these genes, however, in the induction of epithelial tumors which remained at a low incidence, with a variety of target tissues, in congenics as well as NFS/N mice.

Studies of the MCF resistance allele Mcfr. Mcfr is carried by strains DBA/1 and DBA/2 and strains derived from the CBA/H subline of CBA. It is dominant in crosses with sensitive mice and has been mapped to chromosome 5, closely linked (3.3 Cm ± 1.9) to a morphological marker gene, hammer-toe (Hm). It has been found to affect replication of MCF viruses in tissue culture, development of thymic lymphoma in F_1's of AKR and DBA/2 or CBA/N (both spontaneous lymphoma and disease accelerated by inoculation with leukemogenic MCF viruses), and in vivo generation of MCF viruses (either spontaneously as in AKR or following infection of baby mice with leukemogenic ecotropic viruses such as Moloney, Friend, and certain viruses isolated from wild mice). In DBA, CBA/N, or F_1's of these strains with NFS, MCF viruses are not generated and leukemia is much delayed, although ecotropic virus levels are unaffected. In tests of hybrid mice segregating for Mcfr, suppression of susceptibility to the erythroblastic leukemia induced by Friend ecotropic virus was strikingly correlated with presence of Mcfr. Construction of strains of mice congenic for Mcfr (now facilitated by obtaining Mcfr in coupling with Hm) is in progress. Such lines will be used in determining the extent of Mcfr control of various types of murine neoplasia.

Genetic mapping of endogenous viral genes. Eight loci for ecotropic viruses have now been mapped to nonallelic sites on 6 mouse chromosomes. Among the loci mapped recently, Sev-1 was mapped to chromosome 9 in SEA/GnJ, and the V locus of A/J mice was shown to be allelic with that of BALB, a related strain.

Five inbred strains were previously shown to have a single V locus for xenotropic virus, Bxv-1 on chromosome 1. More recently, one inbred strain derived from the same stocks, MA/My, was shown to carry two loci, one of which was identified as Bxv-1.

Since the common inbred strains were derived from colonies including Asian mice, two inbred strains of Asian mice, M. molossinus and M. castaneus, were examined for endogenous V loci. Both were shown to carry multiple ecotropic loci (>2), and spontaneous expression of high titers of ecotropic virus in M. Mol. is under the control of 2 genes. M. Mol. was shown to carry 3 V loci for xenotropic virus.

F/ST mice possess the unusual phenotype of high expression from an early age of both ecotropic and xenotropic virus, the latter being expressed by thymocytes predominantly. Spontaneous expression of xenotropic virus and the xenotropic related cell surface antigen XenCSA is almost completely suppressed in thymocytes of F_1 progeny of F/ST and all other strains tested. Preliminary data strongly suggest that suppression is controlled by a gene in the immune response region. Using IUdR treatment methods to allow scoring for xenotropic virus, a single locus for virus induction has been identified and mapped to chromosome 1, 7 Cm ± 3 from the Pep-3 isozyme locus.

In related studies, the virology of the SJL/J mouse is being investigated. This is a strain with a high incidence of spontaneous B-cell lymphoma and particular emphasis is being placed on the effects of mutant genes affecting the immune system such as lpr and hr.

Heterogeneity of V loci carried in AKR mice. The heterogeneity of endogenous V loci of the AKR mouse suggested by restriction enzyme analysis is now being examined by sexual genetics. The 2 V loci identified in an AKR/N mouse showed linkage to chromosome 7 (Akv-1) and 16 (Akv-2) consistent with previous analysis. However, one AKR/J mouse was shown to carry 3 loci; Akv-1 and 2 novel V loci designated Akv-3 and Akv-4.

Genetics of resistance to Friend disease. The Japanese mouse, M. molossinus, and its hybrids have been analyzed for sensitivity to Friend disease. These mice were shown to carry a dominant resistance gene which appears identical with the Fv-4 locus of the G strain mouse and Japanese wild mice. There is also a second locus on chromosome 5 in these mice which confers an additional resistance to NB tropic Friend virus.

A sexual difference in Friend susceptibility was recently observed in the offspring of SEA/GnJ mothers and is currently being examined.

Pathophysiology of virally-induced myelogenous leukemia. A disease, similar to chronic myelogenous leukemia (CML) in humans, is induced by a retrovirus originally isolated from a wild mouse. Pronounced splenomegaly and anemia with large numbers of circulating immature neutrophils occur after a long latent period. Studies are in progress to establish stem cell perturbations preceding overt disease. Preliminary observations indicate significant preleukemic changes particularly of the erythroid precursors and to a lesser extent of totipotential stem cells. If changes in the preleukemic phase of the disease can be further defined it will represent the first model system available for human myelogenous leukemia. It will be of particular interest to see if uniform time-phased perturbations occur and how these relate to those seen in advanced cases of murine CML.

Classification of murine malignant lymphomas. Neoplasms of the lymphoid system of the mouse have long been classified as those clearly of thymic origin (T cell), lymphatic leukemia and reticulum cell sarcomas. Little attempt has been made to further define this classification using current immunologic concepts including detection of cell surface antigenic markers, internal cytoplasmic immunoglobulin and ultrastructural morphology as well as histology features seen in light microscopy. About 40 spontaneous lymphomas in congenic mice have been examined using methods currently being applied to lymphomas in humans. It has been shown that those murine lymphomas heretofore classified as reticulum cell sarcomas are mainly of B cell origin and many of these are monoclonal. Leukemias resembling human acute or chronic lymphocytic leukemia have also been characterized as B cell lymphomas. Thus it appears that murine lymphomas, particularly those appearing spontaneously in older mice are of B cell origin and of considerable heterogeneity in morphology and external and internal markers.

Molecular biology of endogenous MuLV genomes. A molecularly cloned probe specific for ecotropic MuLVs has been used in conjunction with several restriction enzymes to study the organization of ecotropic-related sequences in the genome of three strains of AKR mice. Three, five, or nine proviral genes have been found in AKR/N, AKR/J, or AKR/Boyse respectively. Only two genes are found in common in all AKR strains. Examination of the DNAs isolated from eight familially related AKR/J mice shows that the set of ecotropic-related proviral genes is relatively stable. Studies in progress are directed toward the determination of which of these genes are expressed and to the possible mechanisms for the strain heterogeneity observed.

A DNA fragment homologous with a portion of a xenotropic MuLV provirus envelope gene has been cloned by recombinant DNA methods. This fragment is being used as a hybridization probe to study the arrangement of related DNA sequences in preparations of different mouse liver DNAs. This probe is also being used to analyze 3 series of interspecific somatic cell hybrids which retain few mouse chromosomes. Preliminary analysis suggests that more than a single provirus is integrated in mouse chromosome 1 in all 3 mouse strains, regardless of whether they carry Bxv-1.

Collaborative studies with N. Hopkins, MIT, are aimed at identifying precise segments of the viral genome which are correlated with specific biological activities of various MCF viruses, particularly leukemogenic activity. Viruses derived from transfections with recombinant DNA's constructed from selected left-hand portions of cloned AKV ecotropic viral DNA and small right-hand pieces of cloned AKR MCF-247 viral DNA are being tested for biological properties distinguishable from the parental ecotropic virus. Preliminary studies indicate that a short segment of the MCF genome may determine the increased ability to infect SC-1 cells, relative to NFS embryo cells, previously found to be characterized of all thymotropic and leukemogenic MCF viruses.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00019-06 LVD

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on the Treatment of Disease with the Interferon System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A complex of poly I·poly C with polylysine has been prepared, without carboxymethyl cellulose. This complex, poly ICL, resists hydrolysis and is able to induce interferon in monkeys to almost the same level as poly ICLC.

Clinical studies with the Interferon inducer poly ICLC have confirmed last year's observations that the drug probably has value in treatment of juvenile laryngo papilloma and certain paralytic neurologic diseases. A clearer understanding has emerged of toxicities induced by the drug, and how to control them.

PHS-6040
(Rev. 10-76)

Achievements

The past year was spent in three activities: 1) the ongoing clinical trials continued and accumulated more patients, 2) we developed and tested a complex of poly I.poly C with polylysine which did not need carboxymethyl cellulose for solubility, and 3) a systematic survey and compilation was done of the effects and toxicity of poly ICLC in the several clinical protocols.

1) Drs. Krown and Oetgen of the Sloan Kettering Institute completed their phase I study of poly ICLC on cancer patients. During this survey, they felt at first, that the pyrogenicity and myalgia seen in some of their patients restricted the acceptable dose to 6-8 mg/m^2. However, through the use of Tylenol, they have been able to administer 12 mg/m^2, as did Levine, et.al. Dr. Krown found, on the two patients with renal cell carcinoma who happened to be among the treated group, that one had a partial remission, and the other had stabilized disease. They plan to submit a protocol for the controlled study of this disease. They also did a study using exogenous interferon, and found about the same type and level of toxicity as they found with poly ICLC.

The Children's Cancer Study Group completed what was to have been a phase II study of the effect of Poly ICLC on acute lymphocytic leukemia in terminally ill children. These children could not tolerate 12 mg/m^2, 6 mg/m^2 being the maximum acceptable. They reversed the designation of their study from phase II to phase I. They found some encouragement in that some bone marrows lost all malignant cells, but the patients died before the marrows regenerated. They are currently starting the phase II aspect of this program.

Drs. Durie and Salmon of the University of Arizona, completed a very short series of patients suffering from advanced multiple myeloma, resistant to other therapy. There were some modest positive effects seen. This group is now engaged in testing exogenous interferon, and will not be returning to poly ICLC very soon.

Dr. Leventhal of Johns Hopkins and Dr. Whisant of the University of North Carolina have looked at the effect of poly ICLC on severe juvenile laryngo papilloma. Of the 8 people treated, 6 are evaluable. In the 9 months preceeding treatment there were 63 surgical interventions. In seven months after treatment there were 5 surgical interventions. They plan to try to do a controlled study.

Drs. Engel and Salazar of the NINCDS have treated thirteen cases of paralytic neurologic diseases. Three did not complete the therapy. Seven patients with peripheral neuropathies responded with good to dramatic improvement. These were patients considered to have demyelinating disease, involving neurones or Schwann cells, probably associated with immune dysfunction. In addition, one patient with severe CNS disease, considered to be an acute attack of MS showed a strong improvement. A protocol for a more extensive study of M.S. is being submitted.

2. In the original description of poly ICLC, it was pointed out that if one added poly lysine to poly ICLC, in physiologic salt, a gummy precipitate was formed, but that if poly lysine was first complexed with carboxymethylcellulose, a soluble complex of poly I.C.poly lysine.CMC could be made. It was thought desirable to be able to element the CMC, because a)there is no known biodegradation path for it and b)the resultant Poly ICLC was a very large molecule. This problem was approached as follows: It was reasoned that precipitation might be occurring because of random cross linking of the PlL with the cooled double stranded Poly I. Poly C. In order to obviate this, poly I and poly C were separately dissolved in H_2O and heated to 60° to remove all 2° structure and to make the molecules adopt a linear form. They were rapidly cooled and mixed. The poly I and poly C would not combine because H-bonding is too weak in water. The plL was added to act as a linking agent between the poly I and poly C, and this reaction allowed to proceed for two days. Finally, salt was added to 0.15 M to allow the d.s. RNA to form. This complex, poly ICL, is quite resistant to hydrolysis and is almost as good an interferon inducer in monkeys as is poly ICLC. It is somewhat less toxic in mice than is poly ICLC.

3. In July of 1980, the CRS requested of the Director, NIAID, that he appoint a committee to review all the clinical studies that have been done with poly ICLC, including toxicity, efficacy and uniformity of results. The CRS requested that all pending protocols be held in abeyance meanwhile. This work has been completed, the report sent to the Director, and new protocols are being prepared to send to the CRS. The report, in essence, said that poly ICLC is able to induce high levels of interferon in people, that it shows marked side effects, probably more severe in extremely ill patients, and much less in patients who are relatively well. The efficacy is discussed in the "Results" Section of this annual report.

Relevance to NIAID and NIH:

Interferon inducers offer the potential of being broad spectrum antiviral and antitumor agents, a goal clearly within the mission of NIH. The immune potentiation possibilities of poly ICLC is directly in line with the mission of NIAID.

Future Plans:

High priority will be given to completing the program, now underway, to develop recording forms that all clinicians will use to record data about their patients. This data will be stored in the NIH computer under a program designed to retrieve information about toxicity, different lots of drugs, beneficial effects, etc.

A number of clinical protocols are being submitted which will include studies of life threatening illnesses associated with viruses.

Efforts will be made to reduce toxicity, including use of adjunct drugs, changes in scheduling and studying poly ICL (without CMS).

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Annual Report
Laboratory of Microbial Structure and Function
Rocky Mountain Laboratories
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1980, to September 30, 1981

RESEARCH HIGHLIGHTS

The Laboratory of Microbial Structure and Function is concerned with defining and understanding structure, function, and control of bacterial components related to virulence and/or pathogenicity of disease-causing organisms. Current studies in LMSF center on the bacterial surface and its role in determining interactions between host constituents and pathogenic bacteria.

In this general context, a number of different organisms are used as "model pathogens" including the following: *Neisseria gonorrhoeae*, *Bordetella pertussis*, several mycoplasmas and spiroplasmas, *Chlamydia trachomatis*, *Coxiella burnetii*, and *Rickettsia rickettsii*. In addition to these, studies on control of bacterial outer membrane protein expression, toxic and other action(s) of lipopolysaccharide, and the occurrences and properties of penicillin-binding proteins are ongoing in LMSF; additional, selected bacteria are the subjects for those experiments. The above-described organisms appear to constitute a rather diverse collection; however, this range of pathogens has proved useful for two major reasons. First, the diversity allows ongoing comparisons of properties of microorganisms whose "pathobiological niches" represent a significant part of the whole spectrum found for bacteria and animal hosts. Obligate intracellular pathogens (*Chlamydia*, *Rickettsia*, and *C. burnetii*), seemingly obligate extracellular parasites (mycoplasmas), and bacteria that exhibit both intra- and extra-cellular existences in the host (gonococci, *B. pertussis*) are represented. Second, the techniques and maneuvers that are initiated or developed in one particular laboratory become of immediate utility and interest to individuals studying similar systems, e.g., surface-exposed proteins, in other organisms. This also promotes direct comparisons of analogous constituents of diverse pathogens. Although the organisms seem diverse, they are all gram-negative, membrane-bounded bacteria and virtually any technique useful for study of one is applicable for studies on the others. This was exemplified by the summer visit of Dr. Carl Frasch (Bureau of Biologics, FDA) who is a well-recognized authority on meningococcal surface components. During Dr. Frasch's stay and his study of group B meningococci at LMSF, he learned several techniques previously applied here for other organisms. He also brought some "tricks" developed for lipopolysaccharide characterization; these have been both used profitably and expanded upon by several of the LMSF staff.

A number of studies carried out during the past year have had significance either to understanding virulence mechanisms of specific organisms and/or in deciphering general strategies used by bacteria in coping with the host millieu.

Acidophilic nature of transport/metabolism in C. burnetii. In the past, it has been puzzling that organisms such as C. burnetii can exist and multiply within the phagolysosome whose conditions would seem, a priori, quite suboptimal for bacterial survival. A seemingly unrelated puzzle has been the past failures to demonstrate significant metabolism of various substrates by C. burnetii, in vitro, under experimental conditions suitable for such demonstrations with Rickettsia typhi. During the past year, Drs. Hackstadt and Williams have provided at least partial explanations for both these riddles by finding that transport, catabolism, and incorporation of several metabolites is optimal under quite acidic conditions, i.e., pH 4.5. This suggests that C. burnetii "prefer" the intraphagolysosomal site since this environment is acidic. Such preference was partially corroborated in experiments using lysotropic agents which "alkalinized" the phagolysosomes and reduced growth of C. burnetii in tissue culture cells. Demonstration of the importance of acid conditions for C. burnetii also provides valuable information relative to formulating an axenic medium for growth of these organisms.

Immunochemical and structural properties of chlamydial outer membrane proteins. Chlamydia are bacteria-like organisms that multiply within host cells in which fusion between phagosomes (containing engulfed Chlamydia) and lysosomes is inhibited. Interruption of this infectious cycle for chlamydiae is probably best accomplished by interference with initial stages such as attachment of organisms to host cells. Work done by Dr. Caldwell during the past year has shown that rabbit antiserum raised against purified major outer membrane protein protects tissue culture cells from chlamydial infection. In the course of that work, relationships between structure and immunochemical characteristics of such proteins from diverse chlamydial serotypes were explored and correlated. These observations are relevant in understanding antibody-dependent neutralization of chlamydial infection of host cells, cross protection by such antibodies, etc.

Surface-exposed gonococcal components. Interactions between gonococci and their human hosts are dictated, at least partially, by those gonococcal components exposed on the organisms' surfaces. Studies carried out during the past year have probed the exposed portions of selected outer membrane proteins to characterize and understand the organism's "personality." Whole, intact gonococci which had been surface radioiodinated were utilized for experiments in which extrinsic proteolysis, immunoprecipitation, or two dimensional display of peptides results provided information about surface exposure of proteins I, II, and III. These studies suggest that gonococci readily alter the "mosaic" of exposed proteins and, thereby, may radically alter both their antigenicities and their liabilities to proteolytic enzymes. Further, surprising degrees of difference were found for the comparative surface exposure of seemingly similar proteins (Swanson and Judd).

Virulence of Bordetella pertussis. B. pertussis has been previously characterized by a myriad of serological, colonial morphology, and other designations in attempts to correlate bacterial properties and the organism's virulence. Production of "pertussigen" is one established virulence-related property, and this material has been recently crystallized by

Dr. Munoz and co-workers. The biological properties of pertussigen have been studied, and the crystalline material seems to account for most virulence-related activities of these complex bacterial pathogens. This material can also induce immunological protection in mice against challenge with B. pertussis. Studies conducted by Dr. Peppler have partially clarified previous observations on the colony forms, serological reactivities, and virulence of B. pertussis. These studies have explored colony morphology, outer membrane protein structure and antigenicities, fatty acid compositions, and elaboration of fimbrial hemagglutinin, pertussigen, and other products for isogenic pairs of organisms. Of particular interest was the finding that a whole complex and seemingly unrelated group of phenotypic characteristics exhibited coordinate "on-off" expression.

Interactions of lipopolysaccharide with lipoproteins and phospholipids. Bacterial lipopolysaccharides display a variety of "endotoxic" reactions on combination with diverse assays such as lethality for chicken embryos, fever production in rabbits, etc. Studies by Dr. McLaughlin during the past year show that some of these endotoxic reactivities can be modified or abrogated by interactions of bacterial lipopolysaccharide with phospholipids or serum lipoproteins. The bases for these interactions which result in alterations of both biological and physical properties of lipopolysaccharide are being investigated.

Microbial penicillin-binding protein. The role(s) played by penicillin-binding proteins in morphology-determination and in action(s) of beta-lactam antibiotics remains murky. The studies started during the past year by Dr. Barbour have provided a number of provocative findings regarding these beta-lactam specific constituents of the bacterial inner or cytoplasmic membrane. Penicillin specifically binds to three distinct proteins in Chlamydia trachomatis. Binding to one of these seems to correlate with penicillin's ability to interrupt intracellular development of infectious elementary bodies from reticulate bodies. These effects are being correlated with penicillin-binding proteins of these two chlamydial forms. The presence and function of such penicillin-binding proteins is of particular interest since these organisms contain no detectable peptidoglycan (Drs. Amano and Caldwell) which is the supposed "target" for penicillin's action. Specific penicillin binding to five proteins has been demonstrated in Borrelia hermsi which undergoes a unique morphologic change at minimal inhibitory concentrations of this antibiotic.

Additional findings. A number of additional findings of note have derived from studies in LMSF during the past year. Staphylococci isolated from vaginae of women with "toxic shock syndrome" were found to differ significantly from vaginal isolates of staphylococci from women with no disease. The most striking correlations were heavy metal resistance, lack of lethality for laboratory animals, and production of an extracellular protein (MW = 22,000, pI = 7.0) by those staphylococci associated with toxic shock (Barbour).

Intracellular development of C. burnetii was studied by electron microscopy and found to consist of several different stages. These include occurrence of an "endospore-like" form which is resistant to physical disruption and may account for the highly resistant, highly infectious nature of *C. burnetii* (Williams).

An *in vitro* model for gonococcal infection utilized explanted fragments of chicken oviducts. These explants were susceptible to damage by gonococci (Hitchcock) and may provide a suitable non-human model for infections by these pathogens. Of additional interest was the finding that both mycoplasmas and gonococci attain higher multiplication levels if they co-infect chicken oviduct fragments as compared with infections with either of the individual organisms (Hitchcock and Liss).

Several facets of the interrelationships between mycoplasmas and their viruses were elucidated. These include the finding that phage-infected spiroplasmas resist physical disruption better than uninfected organisms. No advantage in resistance to lysis was found for acholeplasmas infected with phages. Also, mitomycin C was shown to effect production of phage from several acholeplasmas. For spiroplasmas, phages could not be "induced" by similar treatment (Liss).

Purified *Rickettsia rickettsiae* were subjected to detergent extraction and counter-immunoelectrophoresis techniques to define a group specific antigen for the spotted fever group of rickettsiae. One antigen that is broadly reactive within this group has been identified. Antisera raised against this antigen do not appear to passively protect guinea pigs. Another antigen was found to induce protective immunity to rickettsial challenge; this antigen was absent from typhus rickettsiae and present in 9/14 strains of the spotted fever group. Passive protection of guinea pigs was found for rabbit antisera raised against this antigen, as purified by crossed-immunoelectrophoresis. These and other antigens found in *R. rickettsii* are being utilized for evaluating immune responses in patients with rickettsial infections (Anacker).

Attempts to decipher the mechanisms which control expression of gonococcal outer membrane proteins have been frustrating. Gene banks of the gonococcal genome have been prepared with high copy number plasmids as vehicles; recent evidence suggests that high numbers of certain outer membrane proteins may be lethal for *E. coli*. This and other factors may account for recent failure in selecting transformed clones expressing gonococcal outer membrane proteins. New gene banks are being constructed to reapproach cloning gonococcal genes in *E. coli* or, with additional techniques under development, in an "all-gonococcal" cloning system (Mayer).

Annual Report
Laboratory of Microbial Structure and Function
Rocky Mountain Laboratories
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1980, to September 30, 1981

ADMINISTRATIVE REPORT

Several staff changes occurred in LMSF during the past year. Additions to the scientific staff included Dr. Harlan Caldwell (Expert), Dr. Penny Hitchcock (Staff Fellow), and Dr. Ralph Judd (Staff Fellow). Two Visiting Fellows - Dr. Kenichi Amano and Dr. Mitsuru Watanabe - worked with our scientific staff members. Dr. Carl Frasch (BoB, FDA), Dr. Vincent Fischetti (Rockefeller Univ), Dr. David Walker (Univ of North Carolina), and Ms. Kachiko Sekiya (Kitasato Univ, Tokyo) were Guest Workers. Four college students - Daryl Warder (Yale), Donna Galbreath (Univ of Alaska), Ramona Heiland and Todd Damrow (Univ of Montana) were involved in summer research projects at LMSF. Teresa Brown (BoB, FDA) and Linda Perry (NCI) transferred to join the technical staff. Leaving the technical staff were Mr. Earl Davis, Mr. Richard Kennedy, and Mr. Ron Brown. Departures from the scientific staff included Drs. Edgar Ribi, John Cantrell, and Steven Schwartzman. LMSF and RML were host to the NIAID-sponsored symposium on "Bacterial Virulence and Pathogenesis."

Annual Report
Laboratory of Microbial Structure and Function
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October 1, 1980, to September 30, 1981

HONORS AND AWARDS

Journal Editorial Boards:

J. Swanson - *Journal of Bacteriology* and *Infection and Immunity*

Manuscripts from *Bergey's Manual of Systematic Bacteriology*, *Biochimica et Biophysica Acta*, *Canadian Journal of Microbiology*, *Cancer Immunology and Immunotherapy*, *Infection and Immunity*, *Journal of Infectious Diseases*, *Science*, *Science Books and Films*, and *Sexually Transmitted Diseases* were also reviewed by members of LMSF staff.

Professional Posts:

J. Munoz acted as a trustee of the Stella Duncan Memorial Research Fund at the University of Montana, and was reappointed as Staff Affiliate of Microbiology Department, University of Montana. He served as Chairman of the Immunology Section, ASM, 1980-81. He also organized a symposium on *B. pertussis* and chaired a symposium on *B. pertussis* at the annual ASM meeting.

J. Williams chaired a session (Intracellular Parasites) at the ASM meeting.

A. Liss was a Faculty Affiliate, Department of Microbiology, University of Montana, and a member of masters thesis committee for J. Sanderson, University of Montana. He served as Chairman of the Subcommittee for the Taxonomy of Pseudomonas Bacteriophage, International Committee for the Taxonomy of Viruses.

Invited Lectures:

J. Swanson - ASM annual meeting, Dallas, TX
NIAID Conference on Bacterial Virulence and Pathogenicity

J. Munoz - Society for the Advancement of Chicanos and Native Americans in Science, Albuquerque, NM
ASM annual meeting, Dallas, TX

A. Liss - Cold Spring Harbor Bacteriophage Conference
Washington University School of Medicine, St. Louis, MO

J. Williams - Texas A&M University

H. Caldwell - ASM annual meeting, Dallas, TX

Other Honors:

A. Liss - Juselius Foundation Foreign Scientist Fellowship (Finland)

J. Williams - Research Fellowship, University of Uppsala, Uppsala, Sweden

R. Anacker - Co-editor of Rickettsiae and Rickettsial Diseases, Academic Press, New York (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00065-08 LMSF-E

PERIOD COVERED October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Antigens and Classification of Rickettsiae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Anacker	Res. Microbiologist	LMSF NIAID
	R. N. Philip	Medical Director	EB NIAID
OTHER:	W. J. Todd	IPA Visitor	EB NIAID
	E. A. Casper	Nurse Director	EB NIAID

(See same project number in report of Epidemiology Branch)

COOPERATING UNITS (if any) Dr. K. E. Hechemy, New York State Dept of Health, Albany, NY; Dr. C. M. Wilfert, Duke Univ Medical Center, Durham NC; Dr. K. T. Kleeman, North Carolina State Dept of Health, Raleigh, NC

LAB/BRANCH Laboratory of Microbial Structure and Function and Epidemiology Branch, Hamilton, MT 59840

SECTION

Rickettsial Diseases Section and Epidemiology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	1.5

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are the development of procedures for classification of spotted fever and typhus group rickettsiae and the determination of the nature and biological properties of rickettsial antigens and constituents. From studies utilizing crossed and tandem crossed immunoelectrophoresis, a scheme for the antigenic composition of Rickettsia rickettsii, R. conorii, and R. australis was devised. Localization of an antigen possibly of significance in immunity was accomplished by immunoelectron microscopy. A group antigen was also identified. Antiserum to the group antigen reacted in the micro-immunofluorescence test with representatives of all known serotypes of the spotted fever group. Specificity and persistence of antibodies in sera from Rocky Mountain spotted fever patients were determined by crossed immunoelectrophoresis with an intermediate gel. Two new rickettsial isolates, one representing a widely-distributed serotype, were assigned to the typhus group on the basis of their DNA composition.

Project Description:

Our objectives are to utilize and develop methods for the classification of rickettsiae and to isolate and characterize biologically important antigens of these organisms. Our research is focused principally on rickettsiae of the spotted fever group (SFG) but, because of the recently demonstrated widespread occurrence of rickettsiae of the typhus group (TG) in the U.S., some attention must be given to the latter organisms.

Previously we reported that *Rickettsia rickettsii* has 3 presumed major antigens (1, 2 and 3) and that a monospecific antiserum to antigen 2 prepared in rabbits protected guinea pigs against fever induced by challenge with virulent *R. rickettsii*. Since that report, we have shown with a ferritin-labeling procedure that antigen 2 is located, as we suspected, on the surface of the rickettsiae. Partially purified rickettsiae were incubated first either with the anti-antigen 2 serum or normal rabbit serum and then, after washing, with ferritin-conjugated goat anti-rabbit IgG. Rickettsiae exposed to normal rabbit serum were essentially free of the ferritin label, whereas the surface of rickettsiae incubated with anti-antigen 2 serum was extensively coated with the label. The degree of ferritin labeling was unaffected by inactivation of complement in the immune serum.

Another antigen (1), which is *a* group antigen if not *the* group antigen, has recently been identified. An antiserum to antigen 1 was obtained from rabbits inoculated with immune precipitates prepared by crossed immunoelectrophoresis (CIE) of Triton X-100 extracts of *R. rickettsii*. Purity of the antiserum was demonstrated in 2 ways: (1) the antiserum produced only 1 band of precipitate in a CIE plate charged with a Triton X-100 extract of the whole organism as antigen, and (2) when the anti-antigen 1 serum was incorporated in the intermediate gel of a CIE slide containing the detergent extract of the whole organism as antigen and antiserum to the whole organism in the reference gel, only the band corresponding to antigen 1 was observed in a position different from that noted in the control slide. Evidence that antigen 1 is a group antigen was obtained with the microimmunofluorescence test. Anti-antigen 1 serum reacted with representatives of all 12 SFG serotypes tested at titers ranging from 1:64 to 1:256. This serum did not react with any of the TG rickettsiae. In contrast to the anti-antigen 2 serum, the anti-antigen 1 serum did not prevent death of mice challenged with 2 LD_{50} of *R. rickettsii* toxin.

Our study to compare antigens of *R. rickettsii*, *R. conorii*, and *R. australis* "solubilized" with Triton X-100 was continued. Through CIE analysis of each of the extracts against its homologous antiserum in the reference gel and a heterologous antiserum in the intermediate gel, we were able to devise a scheme for the antigenic composition of these 3 rickettsiae. Sixteen antigens were demonstrated for *R. rickettsii* by this procedure, 9 antigens for R. australis, and 12 for R. conorii. All of the antigens identified for R. conorii and R. australis were also found in R. rickettsii. Only 1 antigen found in the extract of R. rickettsii was absent from extracts of the other 2 SFG agents.

In an attempt to assess the degree of similarity of antigens found in these species, detergent extracts of these rickettsiae were compared by tandem CIE. For reasons not completely understood, the number of reactions of identity or partial identity observed was smaller than expected: 5 for the R. rickettsii-R. conorri comparison and 3 for the R. rickettsii-R. australis comparison. One explanation for the discrepancy may be that the mobilities of some of the related antigens are so different that interaction by tandem CIE is physically impossible.

The guanine plus cytosine content of DNA from 2 rickettsiae isolated from ticks in the U.S. was determined because serologic and other studies indicated that these rickettsiae differed significantly from each other and also from members of the SFG. From DNA thermal denaturation curves, both of these isolates, 1 from California and the other a representative of a serotype distributed throughout the U.S., were found to have a DNA composition like that of the TG agents rather than that of the SFG agents.

Collaboration has continued with Dr. Hechemy of the New York State Department of Health in the development of a latex agglutination test for the serodiagnosis of Rocky Mountain spotted fever (RMSF). Our contribution during this fiscal year has been restricted to providing rickettsial antigen and to serving as an intermediary between Dr. Hechemy and personnel in 9 state health laboratories participating in this double blind study.

We are also still collaborating with Drs. C. M. Wilfert of Duke University and K. T. Kleeman of the North Carolina State Health Laboratories in an epidemiologic study of RMSF. In addition to providing rickettsial antigen for hemagglutination tests, we are collaborating on several different studies. For 1 study, we are examining by CIE sera taken from patients at various intervals after the onset of RMSF to determine (1) whether we can detect antibodies to specific antigens of R. rickettsii, (2) the times of appearance and disappearance of these antibodies, and (3) whether the presence of specific antibodies may be correlated with the course of the disease. A number of sera from individuals with RMSF contracted in 1979 and 1980 were examined. These sera were obtained as early as 2 days and as late as 17½ months after onset of symptoms. Several sera obtained within the first week appeared to have 1 or several kinds of antibodies. One serum obtained at 7 days had antibody to antigen 1. Several sera obtained between 3 to 7 days may have contained antibodies to several other antigens but the reactions were too weak to be certain. Most of the sera obtained from 2 weeks to several months after onset had antibodies to 1 or more antigens, primarily antigens 1, 3, 6, and 7. Antibodies in sera obtained after 16 months were not unequivocally demonstrated. As yet no attempt has been made to correlate the presence of specific antibodies with the clinical course of RMSF in patients.

In a separate but related collaborative study, we examined by CIE with intermediate gel sera obtained recently from individuals who had RMSF up to 40 years earlier (1) to determine persistence of demonstrable antibody and (2) to correlate persistence of antibodies with antibiotic therapy. Sera obtained from 3 individuals who contracted RMSF (1940-42) before the availability of antibiotics had modified responses to antigen 1: the antigen 1 band was found at a greater distance from the origin in these slides than in the control slide. This pattern was not observed in tests with sera from 4 patients infected (1951-75) after the advent of antibiotics. Also, 2 of the 3 former sera had easily demonstrated antibodies to antigen 3; barely demonstrable levels of anti-2 antibodies appeared to be present in these 3 sera.

The CIE patterns of sera obtained from the individuals infected after 1950 differed in other ways from the patterns of sera from patients infected in the 1940-42 era. Two of the 4 had questionable levels of antibodies to 2 and 3, and the other 2 sera had no antibodies detectable by our CIE technique. It appears from this limited sampling that antibiotic therapy may suppress levels and kinds of _R. rickettsii_ antibodies normally persisting after infection.

Thus far we have obtained a modest amount of information about 3 possibly important antigens of _R. rickettsii_, antigens 1, 2, and 3. (Although our information about immunity is restricted to humoral immunity, we recognize that cellular factors may be very important in the host's response to infection.) Antigen 1 may not be important as an immunogen since antiserum to this antigen failed to protect mice, but the presence of serum antibodies to this antigen both early and late suggests that this antigen merits further study. In addition, a monospecific antiserum to antigen 1 may serve as a convenient tool for rapid identification of SFG agents. Antigen 2 is still a candidate for a subcellular vaccine since its surface location provides immediate accessibility to the host's defense factors and because we have demonstrated that anti-antigen 2 antibodies protect both guinea pigs and mice from _R. rickettsii_. Antigen 3 also deserves further study. The presence of serum antibody to this antigen during the convalescent stage and at least for 40 years after infection indicates a significant response to this antigen by the host.

In the future we will continue our attempts to purify and characterize potentially important antigens of _R. rickettsii_. We particularly would like to isolate antigens 2 and 3 so that we can define the humoral and cellular responses of mice and guinea pigs to these antigens. Also, we will examine more sera from RMSF patients by CIE. Some concepts about the antibody responses of patients to RMSF have emerged, but additional data are necessary to adequately describe these responses. In a serum survey evidence which suggested the possibility of exposure of humans to other SFG agents prevalent in ticks was obtained; we will examine these sera by CIE in an attempt to determine whether the antibody response was actually directed against rickettsiae other than _R. rickettsii_. Finally, we have embarked on a study to identify and characterize surface antigens associated with virulence of _R. rickettsii_.

Publications:

Hechemy, K. E., Anacker, R. L., Philip, R. N., Kleeman, K. T., MacCormack, J. N., Sasowski, S. J., and Michaelson, E. E.: Detection of Rocky Mountain spotted fever antibodies by a latex agglutination test. _J. Clin. Microbiol_. 12: 144-150, 1980.

In press:

Wilfert, C. M., Austin, E., Dickinson, V., Kleeman, K. T., Hicks, J. L., MacCormack, J. N., Anacker, R., Casper, E., and Philip, R. N.: The incidence of Rocky Mountain spotted fever as described by prospective epidemiologic surveillance and the assessment of persistence of antibodies to _R. rickettsii_ by indirect hemagglutination (IHA) and microimmunofluorescence (MIF) tests. In Burgdorfer, W. and Anacker, R. L. (Eds.): _Rickettsiae and Rickettsial Diseases_. New York, Academic Press.

Kleeman, K. T., Hicks, J. L., Anacker, R. L., Philip, R. N., Casper, E. A., Hechemy, K. E., Wilfert, C. M., and MacCormack, J. N.: Early detection of antibody to _Rickettsia rickettsii_: A comparison of four serological methods, indirect hemagglutination, indirect fluorescent antibody, latex agglutination, and complement fixation. In Burgdorfer, W. and Anacker, R. L. (Eds.): _Rickettsiae and Rickettsial Diseases_. New York, Academic Press.

Hechemy, K. E., Sasowski, S. J., Michaelson, E. E., Zdeb, M., Anacker, R. L., Philip, R. N., Kleeman, K. T., Crump, C., Kudlac, J., Joseph, J. M., Patel, J., Dowds, H. Jr., Killgore, G., Young, D., Barrick, J. H., Hindman, J. R., Elliott, L. B., and Altieri, R. H.: Rocky Mountain spotted fever: First-year evaluation of a latex agglutination test for antibodies to _Rickettsia rickettsii_. In Burgdorfer, W. and Anacker, R. L. (Eds.): _Rickettsiae and Rickettsial Diseases_. New York, Academic Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00070-16 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biological Activities of Substances in *Bordetella pertussis*

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. K. Bergman Scientist Director LMSF NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
Pertussis Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00071-11 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Purification and Activities of Biologically Active Substances from Bordetella pertussis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. J. Munoz	Res. Microbiologist	LMSF NIAID
OTHER:	M. Watanabe	NIH Visiting Fellow	LMSF NIAID
	K. Sekiya	Guest Worker	LMSF NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

Pertussis Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 5.8	PROFESSIONAL: 2.5	OTHER: 3.3

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are (1) to characterize the biologically active substances from Bordetella pertussis, (2) to elucidate the mechanisms of action of these substances, and (3) to develop an effective, purified, nontoxic vaccine for whooping cough. This project emphasizes the purification and mode of action of pertussigen and other substances thought to be involved in immunizing man against whooping cough. The mechanisms of action are emphasized because pertussigen induces changes in mice that have counterparts in diseases of man. For example, pertussigen induces a marked increase in the production of IgE antibody, it increases the susceptibility to experimental allergic encephalomyelitis (EAE), it produces a marked lymphocytosis and stimulates the production of insulin. In addition, pertussigen increases susceptibility to endotoxic and anaphylactic shock and decreases resistance to various types of stress. The understanding of how pertussigen induces these changes may shed some light into how certain diseases in man (such as allergies, neurologic disorders, diabetes, excess proliferation of lymphocytes and shock) may be controlled.

Project Description:

The main objectives are (1) to fully characterize biologically active substances from Bordetella pertussis (pertussigen, fimbrial hemagglutinin agglutinogens and dermonecrotic toxin), (2) to find the mechanisms of action of these biologically active substances, and (3) to develop an effective nontoxic purified vaccine for whooping cough.

1. Purification of pertussigen (Muncz). A great deal of time has been spent growing B. pertussis and preparing semi-purified pertussigen for use in further purification studies of this substance. A convenient method has been adapted as routine for the production of this crude pertussigen: B. pertussis is grown in 500 ml of synthetic liquid medium of Steiner-Scholte contained in 4-liter Erlenmeyer flasks and incubated for 5 days at 35°C. The cells are removed by centrifugation. The supernatant fluid is adjusted to pH 6.5 with acetic acid, and then enough $ZnCl_2$ is added to bring the final concentration to 10 g per liter. The precipitate formed is collected and resuspended in 37 ml (per each liter of culture supernatant) of 10% Na_2HPO_4, stirred, centrifuged, and the supernatant fluid saved. The sediment is extracted twice with 37 ml of 50 mM Tris in 1 M NaCl, pH 8. The extracts are pooled with the Na_2HPO_4 extract. This extract is highly active in sensitizing mice to histamine. It can be dialyzed extensively against water and lyophilized to store as a stable powder. One such preparation was active at 4 ng per mouse. The technique has been used with different strains of B. pertussis with equal success.

We have done work on the removal of trace contaminants of purified preparation of pertussigen. One method which is promising is the use of hydroxylapatite column chromatography to remove FHA from pertussigen. This technique was developed by J. Cowell et al. at the BoB.

2. Biological activities of pertussigen (Munoz, Bergman). Crystalline pertussigen was tested for its various activities, and the results are outlined in the following table:

Biological Activities of Pertussigen in Mice

Activity	Dose in ng protein
Histamine-sensitizing (SD_{50})	0.5
Leukocytosis-promoting (effective dose)	8.0
Stimulation of insulin (effective dose)	2.0
Toxicity (LD_{50})	546.0
Increased permeability in muscle (effective dose)	2.0
Increased production of IgE (effective dose)	0.1
Increased anaphylactic sensitivity (SD_{50})	10.0
Protects mice from i.c. challenge with B. pertussis (PD_{50}) (after detoxification with glutaraldehyde)	1700.0
Experimental allergic encephalomyelitis in Lewis rats (effective dose)	20.0

We also found that the histamine sensitizing effects of crystalline pertussigen lasted longer than 84 days after an i.v. administration of as little as 0.5 ng.

3. Characterization of pertussigen (Munoz). Pertussigen has been solubilized in a formic-acid buffer containing alpha-alanine (0.287 ml formic acid; 3.57 g alpha-alanine in 1 liter of water, pH 3.5). In this buffer pertussigen seems to give two distinct major protein bands in acrylamine gel electrophoresis. When dissolved in acetic acid buffer (31.2 g beta-alanine, 8 ml glacial acetic acid, pH 4.5), it gives only one major band. Crystalline pertussigen gives four major and one minor protein bands in SDS-PAGE. The four major bands are similar to those seen by Irons and MacLennan and Morse in their purified preparations. Others have found only three protein bands by SDS-PAGE. We are continuing this work.

4. Preparation of anti-pertussigen sera (Munoz). Anti-pertussigen has been prepared in rabbits by giving one s.c. injection of pertussigen emulsified in CF adjuvant and two weeks later an injection of pertussigen into the popliteal lymph nodes. Two weeks later the rabbits were bled and the sera collected and tested for antibodies to pertussigen and FHA. Some of the rabbits developed what appeared to be specific antiserum. However, when these sera were tested against crude extracts of the Sakairi strain of B. pertussis which does not produce pertussigen, a prominent precipitin band developed. This band could be easily absorbed by whole cells of Sakairi strain, leaving specific antibody for pertussigen.

5. Studies on the heat-labile dermonecrotic toxin (Sekiya and Munoz). The dermonecrotic toxin (DNT) is a highly toxic protein from B. pertussis. It induces necrotic lesions on the skin of guinea pigs and rabbits but not in adult mouse skin. It has a pronounced degenerative effect on the spleen of mice and possibly also on the lymph nodes and thymus. For these reasons we are studying the effect of DNT on antibody formation. DNT is a very unstable substance losing its toxicity within a few days at 2 to 5°C. In the presence of 20 mM sucrose, it is more stable. We have made toxic solutions by disrupting cells by ultrasonic sound. From these solutions, clarified by centrifugation, partially purified toxin was made by passing the crude toxin through a DEAE-sephacel column equilibrated in 0.01 M phosphate buffer, pH 8, containing 20 mM sucrose. The toxin was absorbed by the DEAE-sephacel. The column was then flushed successively with 0.05 M phosphate pH 7.2 buffer and 0.2 M phosphate pH 7 buffer. All the buffers contained 20 mM sucrose. The toxic fractions eluted by the last buffer were pooled, concentrated and dialyzed against 0.005 M phosphate buffer containing 20 mM sucrose to precipitate pertussigen and FHA which were present as contaminants. The clarified toxic solution was then passed again through a DEAE-sephacel and eluted as above. This rechromatographed toxin was used in the present studies.

Preliminary results indicate the following:

(a) Toxin given simultaneously with antigen (egg albumin) plus pertussigen does not reduce the production of IgE antibodies in mice. The results indicate an actual increase in IgE anti-egg albumin titers. On the other hand, the titers of IgG_1 antibody were slightly lower.

(b) Toxin and pertussigen given five days before antigen did not reduce the IgE production but reduced the IgG_1 titers in C57BL/10 mice.

(c) Toxin markedly reduced the spleen weight of C57BL/10 mice for over 28 days (maximum length of time observed). In CFW mice most of the spleens had returned to normal weight by the 28th day.

6. Mutants of *B. pertussis* that do not produce FHA (Watanabe and Munoz). Dr. M. Watanabe has developed by U-V irradiation mutant strains of *B. pertussis* that have lost their ability to produce FHA, but still retain their ability to produce pertussigen and other factors associated with smooth (Phase I, dome hemolytic) strains. We have compared a number of strains, including two U-V mutants, for their various biological properties such as (a) histamine sensitizing, (b) lymphocytosis promoting, and (c) mouse protecting and hemagglutinating activities. The results are tabulated in the following table:

Activities of UV Mutants Compared to Standard Strains

Strain		FHA in 1 M Na-acetate extract	FHA by gel diffusion test	HSF LD_{50} millions	LPF	Mouse protection ED_{50} millions
Designation	Agglutinogen type					
18-323	1,2,4	8	+	.9	+	32.4
UV-1544	1,2,4	-	-	4.5	+	52.5
UV-1550	1	-	-	2.8	+	89.2
3779 BL_2S_4	1,3,6	1024	+	ND	ND	ND
2753	1,2,3,4	32	+	ND	ND	ND
Maeno	1,2,3,4,6	2048	+	ND	ND	ND
Tohama	1,2,4	2048	+	ND	ND	ND
Yamaguchi	1,3,6	1024	+	ND	ND	ND
Nakano	1	64	+	3.2	+	20.0
Sakairi	none	-	-	ND	ND	ND

From these results it is clear that the two mutants obtained by UV irradiation (UV-1544 and UV-1550) do not produce FHA, but they produce pertussigen and protect mice from intracerebral challenge. The results also show that among smooth (Phase I, dome-hemolytic) strains there are quantitative differences in their production of FHA and pertussigen.

We are now determining whether some of the properties of strains of B. pertussis are associated with the presence of a particular plasmid.

Future work. In 1982 the principal investigator (JJM) will be on special assignment at the Walter and Eliza Hall Institute for Medical Research in Melbourne, Australia, where he will concentrate on studies on the mechanism of action of pertussigen with respect to its acceleration of experimental allergic encephalomyelitis and its effects on experimental vaccines for parasitic infections. The operation of the pertussis section at the Rocky Mountain Laboratories will be discontinued until my return.

In the past two years the pertussis section has been gradually reduced to its present size consisting of the principal investigator, three technicians, a visiting fellow and a guest worker. Two of the three technicians were transferred from Dr. Ribi's section upon his retirement. One of these two technicians will retire in August. During my absence there will be no qualified professional person to utilize the two technicians left, and they should be reassigned to other investigators within the laboratory. Upon my return in 1983, new directions in the pertussis projects may be developed, depending on new findings and on support from the laboratory.

Miss Sekiya, who has been a guest worker since December 1980, will return to Japan in August 1981, and Dr. Watanabe, who has been with us as a visiting fellow since February 1981, plans to return to Japan at the end of January 1982.

Publications:

Arai, H. and Munoz, J. J.: Crystallization of pertussigen from Bordetella pertussis. Infect. Immun. 31: 495-499, 1981.

Munoz, J. J., Arai, H., and Cole, R. L.: Mouse-protecting and histamine-sensitizing activities of pertussigen and fimbrial hemagglutinin from Bordetella pertussis. Infect. Immun. 32: 243-250, 1981.

In press:

Munoz, J. J., Arai, H., Bergman, R. K., and Sadowski, P. L.: Biological activities of crystalline pertussigen from Bordetella pertussis. Infect. Immun.

Munoz, J. J., and Arai, H.: Studies on crystalline pertussigen. Proceedings of International Symposium on Bacterial Vaccines, September 15-18, 1980.

Munoz, J. J.: Biological activities of B. pertussis antigens. Proceedings of Society for Advancement of Chicanos and Native Americans in Science Meeting, November 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00076-23 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immunopotentiation by Components of Microbes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. A. McLaughlin	Senior Staff Fellow	LMSF NIAID

COOPERATING UNITS (if any)

Dr. L. F. Woodard, Dept Vet Med, U of Idaho, Moscow, ID

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to delineate, at the cellular and molecular levels, the mechanism of action of microbial components in immunoregulation. In the past year the main research activities have been investigations of the interaction of endotoxin with lipoproteins of human plasma and with phospholipids.

PHS-6040
(Rev. 10-76)

Project Description:

Several related studies published within the last two decades demonstrate that endotoxin from a variety of gram-negative bacteria interact with phospholipids of eukaryotic cells and with plasma components. In addition, modification of endotoxic biological activity by serum or plasma components has been observed by a number of investigators. However, no one has reported modification of endotoxic activity by phospholipids. Since plasma lipoproteins contain appreciable quantities of phospholipids, I have studied the physical-chemical interaction of endotoxins with lipoproteins and phospholipids and the biological effects of these interactions.

The amphipathic properties of endotoxin and phospholipids appear to serve as the impetus for the interaction of these two classes of molecules in the cell wall of gram-negative bacteria, in the endotoxic extracts prepared from these bacteria, and in eukaryotic systems. I have found that phospholipids present in extracts containing endotoxin are noncovalently attached to endotoxin molecules. Physical-chemical studies demonstrated that this interaction affects the apparent physical properites (density) and apparent chemical composition (fatty acid composition) of the endotoxin. In addition, liposomes made of phospholipids and endotoxin lack endotoxic activity as measured by chick embryo lethality but retain pyrogenic activity in rabbits. Thus, interaction of phospholipids and endotoxin affect the apparent physical-chemical and biological activity of endotoxin.

Modification of the physical nature and biological activity of endotoxin by components of human plasma was found similar to modification of endotoxin by phospholipids. Isolated lipoprotein fractions of human plasma bind radiolabeled endotoxin thereby causing the density of the endotoxin to shift from a high value to a low value equal to the lipoprotein. The process is a two-step one consummated by abrogation of endotoxicity in the chick embryo lethality assay. The first step involves unidentified protein(s) that probably cause disaggregation of the endotoxin molecules. The second step is the interaction of the endotoxin or endotoxin-protein complexes with very low, low, and high density lipoproteins. This process is reversible, heat-resistant, and dependent upon endotoxin, plasma protein, and lipoprotein concentration. The process is sensitive to pronase treatment provided the plasma components are treated with pronase before incubation with endotoxin. However, the final endotoxin-protein-lipoprotein complex is resistant to pronase digestion but can be disaggregated by organic solvent extraction. It appears that the endotoxin molecules associated with the plasma protein(s) and lipoproteins are not recognized or are processed differently by the chick embryo than are unmodified endotoxins.

As a continuation of last year's collaborative studies with Dr. Lynn Woodard, we compared the adjuvant activity of three chemically defined substances in augmenting resistance to _Brucella abortus_ infection. Guinea pigs immunized with test adjuvants and/or heat-killed whole cells of a rough strain of _B. abortus_ were challenged with a virulent strain of _B. abortus_.

We found that addition of trehalose dimycolate to the whole cells was as effective as complete Freund adjuvant in augmenting resistance to *B. abortus* infections. Muramyl dipeptide and dimethyl dioctadecyl ammonium bromide were not effective adjuvants. Studies are in progress to ascertain if the trehalose dimycolate-whole cell vaccine will provide protection in cattle against abortion caused by *B. abortus* infections.

The future course of this project for the ensuing year will be concentrated on (1) determining the consequence of interaction of endotoxin with phospholipids and lipoproteins by examining effects of these complexes on eukaryotic cells and (2) ascertaining if endotoxin will stimulate phospholipid-dependent enzymes such as protein kinase or phospholipase *in vitro*.

Publications:

Woodard, L. F., Toone, N. M., and McLaughlin, C. A.: Comparison of muramyl dipeptide, trehalose dimycolate, and dimethyl dioctadecyl ammonium bromide as adjuvants in *Brucella abortus* 45/20 vaccines. *Infect. Immun.* 30: 409-412, 1980.

Woodard, L. F., Toone, N. M., and McLaughlin, C. A.: Immunogenic properties of soluble antigens or whole cells of *Brucella abortus* strain 45/20 associated with immunoadjuvants. I. Soluble antigens. *Can. J. Comp. Med.* 44: 453-455, 1980.

Woodard, L. F., Toone, N. M., and McLaughlin, C. A.: Immunogenic properties of soluble antigens or whole cells of *Brucella abortus* strain 45/20 associated with immunoadjuvants. II. Whole cells. *Can. J. Comp. Med.* 44: 456-458, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00077-25 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure and Biological Activity of Endotoxins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
OTHER:	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
	S. M. Schwartzman	Senior Staff Fellow	LMSF NIAID
	K. Amano	NIH Visiting Fellow	LMSF NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

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SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00078-09 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Synthesis, Biological Activity, and Immunotherapeutic Activity of Microbial Constituents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. M. Schwartzman	Senior Staff Fellow	LMSF NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00087-04 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunological Activity of Eukaryotic and Prokaryotic Cellular Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
	S. M. Schwartzman	Senior Staff Fellow	LMSF NIAID

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00182-03 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical and Genetical Mechanisms of Obligate Intracellular Parasitism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. C. Williams	Scientist	LMSF NIAID
OTHER:	T. Hackstadt	Staff Fellow	LMSF NIAID

COOPERATING UNITS (if any)

Dr. Thomas McCaul, Dept Med Microbiol, London School of Hygiene and Trop Med, Keppel Street, London WCIE 7HT, England

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SECTION

Rickettsial Diseases Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2.2	PROFESSIONAL: 1.3	OTHER: 0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective is to characterize the biochemical and genetical mechanisms of obligate intracellular parasitism between the eukaryotic host and prokaryotic parasite. Molecular interactions at the cell surface and with soluble components are being investigated as primary determinants of metabolic cooperation between host and parasite. An insight into the biochemical strategy of obligate intracellular parasitism of phagocytic cells has been achieved by studying the in vitro metabolic capabilities and in vivo effect of lysosomotropic amines on the growth of Coxiella burnetii. A pathogenic mechanism based on regulation of microbial metabolism by H^+-dependent stimulation of cell function has been described for C. burnetii, the etiologic agent of Q fever. This organism is a gram-variable and sporogenic obligate intracellular parasite which multiplies only in the phagolysosome of eukaryotic cells. Thus, a key feature of phagolysosomal growth of C. burnetii is a membrane structurally and functionally compatible with an otherwise hostile environment. Our recent studies support the hypothesis that intracellular parasites have evolved metabolic programs complementary to the cellular compartment in which they reside.

PHS-6040
(Rev. 10-76)

Project Description:

The molecular basis of bacterial obligate intracellular parasitism is being investigated in a conceptual framework employing pathogenic bacteria coinjoined to form a continuum based on regulation of microbial metabolism. Fundamental common characteristics for facultative and obligate intracellular bacteria are their ability to (1) avoid destruction by the microbicidal environment and (2) to metabolize common substrates provided by the highly compartmentalized eukaryotic host. More important, a predominate feature of intracellular growth is the highly specialized pathogenic mechanism of phagosomal, phagolysosomal,and cytoplasmic pathogens. Those bacteria which simply avoid destruction within eukaryotic cells are not considered in this model since these bacteria are generally in bacterial stasis during their sojourn through the host cell. Thus, we are interested in a variety of parasitic mechanisms which may be protective or aggressive responses to host defense mechanisms.

Coxiella burnetii, the etiologic agent of Q fever, is a gram-negative spore-forming obligate intracellular parasite of eukaryotes. Unlike the majority of successful bacterial parasites which escape the bactericidal environment of the phagolysosome by various means, *C. burnetii* multiplies only in the phagolysosome. In view of the relatively harsh environment inhabited by *C. burnetii*, we have examined (1) the *in vitro* metabolism of glucose and glutamate by whole cells of *C. burnetii* under conditions designed to approximate the pH within the phagolysosome and (2) the effect of manipulation of the phagolysosomal pH by lysosomotropic amines on the replication of *C. burnetii* in chicken embryo fibroblasts. The transport, catabolism, and incorporation of both glucose and glutamate were found to be highly stimulated by acidic conditions, whereas, at pH 7.0 metabolism of these substrates was minimal. The transport processes were shown to be energy dependent and highly sensitive to inhibition by uncouplers of oxidative phosphorylation. Increasing the phagolysosomal pH of infected chicken embryo fibroblasts by use of the lysosomotropic agents chloroquine, methylamine, or ammonium chloride inhibited the multiplication of *C. burnetii* thus demonstrating the *in vivo* requirement for the acidic conditions of the phagolysosome. This apparent dependence upon phagosome-lysosome fusion to generate pH conditions favorable to *C. burnetii* replication suggested a unique biochemical mechanism of parasite activation. A pathogenic mechanism based on regulation of microbial metabolism by H^+-dependent stimulation of cell function was proposed. This mechanism of activation does not appear to be a general feature of obligate intracellular parasites as demonstrated by the neutral pH optima for metabolism by *Rickettsia typhi* and *Chlamydia psittaci*. Of the substrates examined, all were catabolized and/or incorporated better by *C. burnetii* under acidic conditions than at neutrality. In addition, precursors of both DNA and RNA were incorporated by *C. burnetii* at pH 4.5 in the presence of an energy source, thus implying the generation of a sufficient energy charge to drive macromolecular synthesis in an axenic medium.

Since the demonstration of the activation of C. burnetii metabolism by pH conditions representative of the phagolysosome, a major effort has been directed toward understanding the molecular basis of this activation with the ultimate goal being a description of the triggering mechanism for differentiation and growth of this organism. Since growth of C. burnetii has not yet been demonstrated in an extracellular environment, a number of techniques have been investigated as indicators of metabolic activation of intact C. burnetii under defined conditions.

The ability of C. burnetii to couple oxidation of metabolic substrates to ATP synthesis in axenic reaction buffers was examined. Pyruvate, succinate, and glutamate, respectively, were found to be both catabolized and incorporated at the highest rates of 11 substrates tested. Glutamate oxidation, however, resulted in the greatest stability of the ATP pool and highest intracellular ATP levels over a 48 h period. In a reaction buffer of pH 4.5, the optimum for metabolism by C. burnetii, glutamate oxidation resulted in maintenance of the ATP pool at a concentration of approximately 0.7 nMol ATP/mg dry wgt over a 96 h period. In the absence of substrate, ATP declined by 96 h to less than 0.01 nMol/mg dry wgt. When cells were maintained in a reaction buffer of pH 7.0 in the presence or absence of glutamate, ATP pools were considerably more stable, presumably due to the minimal metabolic activity displayed by C. burnetii at pH 7. The stability of the ATP pool reflected viability as there was greater than an 8-log decrease in viable C. burnetii following incubation for 7 days at pH 4.5 in the absence of glutamate. Viability was retained, however, when the cells were provided with glutamate at pH 4.5 or at pH 7.0 in the absence of any added substrate. The stability of the ATP pool was due to endogenous synthesis of ATP coupled to substrate oxidation as shown by depression of ATP levels in the presence of inhibitors of electron transport or oxidative phosphorylation. In addition, the adenylate energy charge increased from an initial value of 0.57 to 0.73 during glutamate oxidation with a concomitant rise in the total adenylate pool size. C. burnetii therefore appears able to regulate endogenous ATP levels in response to substrate availability and pH, thus affecting a conservation of metabolic energy in neutral or alkaline environments.

Because of the acidophilic nature of C. burnetii, the magnitude of the total proton motive force, which consists of both a transmembrane pH gradient, Δ pH (inside alkaline) and electrochemical potential $\Delta\psi$ (inside negative), was examined. The intracellular pH of non-respiring C. burnetii is relatively stable at about pH 5.8 over a range of extracellular pH of 2-6 and increases slightly at pH values greater than 6.0. Over the pH range of active metabolism by C. burnetii (pH 3-5.5), the intracellular pH increased by up to 1 unit and membrane potential by up to 20 mV in the presence of metabolizable substrate. This increase is Δ pH and $\Delta\psi$ was inhibited by uncouplers of oxidative phosphorylation such as carbonylcyanide-m-chlorophenyl ketone. These phenomena take place within about 5 min exposure of the organisms to appropriate conditions and a number of substrates and do not increase further after up to a one h incubation at 37°C. Thus membrane activation, required for such processes as ATP synthesis and energization of transport processes, appears to be one of the immediate responses of C. burnetii to acid pH and available substrate. Such mechanisms have been proposed to play a role in the resistance of C. burnetii to environmental conditions and subsequent activation upon entry into the normally microbicidal phagolysosome in which this organism replicates.

The future course of this project will center around four areas as specified below: (1) Since we have discovered the acidophilic nature of C. burnetii, a major portion of our time will be spent on describing the metabolic basis of energy transformation. (2) A thorough search will be made for the proper conditions to grow C. burnetii in an axenic medium. (3) The genetic basis of the phase transition of C. burnetii will be studied with the highly purified microorganisms by attempting to transfer genetic material from phase I cells to phase II cells. (4) Utilization of host nucleotide pools by R. typhi in selected tissue culture strains will be examined. An attempt will be made to correlate rickettsial growth with nucleotide availability.

Publications:

Weiss, E., Peacock, M. G., and Williams, J. C.: Glucose and glutamate metabolism of Legionella pneumophila. Curr. Microbiol. 4: 1-6, 1980.

Hackstadt, T. and Williams, J. C.: Biochemical stratagem for obligate parasitism of eukaryotic cells by Coxiella burnetii. Proc. Natl. Acad. Sci. USA 78: 3240-3244, 1981.

In press:

Hackstadt, T. and Williams, J. C.: Stability of the adenosine triphosphate pool in Coxiella burnetii: Influence of pH and substrate. J. Bact.

McCaul, T. F., Hackstadt, T., and Williams, J. C.: Ultrastructural and biological aspects of Coxiella burnetii under physical disruptions. In Burgdorfer, W. and Anacker, R. L. (Eds.): Rickettsiae and Rickettsial Diseases. New York, Academic Press.

Spicer, A. J., Peacock, M. G., and Williams, J. C.: Effectiveness of several antibiotics in suppressing chick embryo lethality during experimental infections by Coxiella burnetii, Rickettsia typhi and R. rickettsii. In Burgdorfer, W. and Anacker, R. L. (Eds.): Rickettsiae and Rickettsial Diseases. New York, Academic Press.

Hackstadt, T. and Williams, J. C.: Incorporation of macromolecular precursors by Coxiella burnetii in an axenic medium. In Burgdorfer, W.

and Anacker, R. L. (Eds.): Rickettsiae and Rickettsial Diseases. New York, Academic Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AI 00183-03 LMSF

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Structural and Functional Relationships of Bacterial Antigens in the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. C. Williams	Scientist	LMSF NIAID
OTHER:	T. Hackstadt	Staff Fellow	LMSF NIAID
	M. G. Peacock	Microbiologist	EB NIAID
	T. A. Damrow	Graduate Student	LMSF NIAID
	K. Amano	NIH Visiting Fellow	LMSF NIAID
	L. A. Thomas	Research Biochemist	EB NIAID

COOPERATING UNITS (if any) Drs. E. Weiss and G. Dasch, Naval Med Res Inst, Bethesda, MD; Dr. C. O. Kindmark, Act Head Dept Infectious Diseases, University Hosp, S-75014 Uppsala, Sweden; Dr. T. McCaul, London School of Hygiene and Trop Med, England; Dr. F. T. Koster, Div Infectious Dis, Univ of New Mexico School Med, Albuquerque

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
Rickettsial Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.2	1.7	3.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective is to characterize the protective antigens of the rickettsiae and other selected bacteria which are pathogenic for humans. Structure and functional aspects of the immunomodulation by the cell wall and soluble components of Coxiella burnetii (Cb) are currently being analyzed as potential candidates for subunit vaccines. We have shown that (1) whole cell vaccines of Cb suppress the normal mitogenic response of spleenic lymphocytes to ConA, PHA, and PWM, but a subfraction of Cb did not induce this suppression; (2) at least six surface proteins of Cb are recognized by immune sera from rabbits, guinea pigs, and mice; (3) Cb releases a soluble antigen which forms a circulating immune complex in the sera of infected guinea pigs; and (4) endospore formation is characteristic of Cb during its growth in the phagolysosome of host cells.

Project Description:

Modulation of the immune response during bacterial infection can produce activation or suppression of a variety of humoral or cellular factors. Components on bacterial surfaces and those secreted during infection mediate the normal immune mechanisms. The objective of this project is the elucidation of the chemical structures required for positive or negative immunomodulatory activities of microorganisms pathogenic for humans. Our goal is the establishment of experimental procedures for analyzing components of bacteria in animals and _in vitro_ models. Of primary importance is the characterization of subfractions of _Coxiella burnetii_ which are important for immunological efficacy in the mouse, guinea pig, and rabbit before subjecting the fractions to clinical evaluation in humans. The ultimate aim is the elucidation of structural and functional relationships between biological response modifiers.

C. burnetii is a gram-variable obligate intracellular bacterium which carries out its development cycle in the phagolysosome of eukaryotic cells. Ultrastructural analysis of _C. burnetii_, _in situ_ and after Renografin purification, by transmission electron microscopy of lead-stained thin sections has revealed extreme pleomorphism as demonstrated by two morphological cell types, a large cell variant (LCV) and a small cell variant (SCV). Potassium permanganate staining of purified rickettsiae revealed a number of differences in the internal structures of the cell variants. (1) The outer membrane of the SCV and LCV were comparable; however, the underlying dense layer of the SCV was much wider and more prominent than that of the LCV. The periplasmic space of the SCV was not readily visualized, whereas, the periplasmic space of the LCV was apparent and resembled that of other gram-negative bacteria. (2) Complex internal membranous intrusions which appeared to originate from the cytoplasmic membrane were observed in the SCV. The LCV did not harbor an extensive membranous system. (3) Some LCVs contained a dense body in the periplasmic space. This endogenous structure appeared to arise in one pole of the LCV as an electron-dense "cap" formation with the progressive development of a dense body approximately 130 to 170 nm in diameter which was eventually surrounded by a coat of at least four layers. Our observations suggest that the morphogenesis of _C. burnetii_ is comparable, although not identical, to cellular differentiation of endospore formation. A developmental cycle consisting of vegetative and sporogenic differentiation was proposed.

Surface proteins probably play dominant roles in the mosaic of antigenic determinants prominent on particular cells. The role of these components as antigenic components, in immunological recognition, as factors in the host-parasite interaction, (i.e., complementary surface structures), as mediators of metabolic events and as virulence factors are not understood for Phase I _C. burnetii_. Phase II _C. burnetii_, which is generated by repeated passage in embryonated eggs, is a spontaneous mutant of _C. burnetii_, and it is not virulent for guinea pigs and mice. Preliminary studies employing Phase II _C. burnetii_ have shown that this avirulent strain has retained only a few of the surface proteins of Phase I cells. Therefore, a comparison of the surface proteins of these two phases of the same strain of *C. burnetii* may lead to a better understanding of bacterial virulence.

A long range objective is the characterization of the membrane components unique to *C. burnetii* cells. Iodination of *C. burnetii* cells was catalyzed in a mixture containing lactoperoxidase, iodide, phosphate-buffered saline (pH 7.3), sucrose, carrier-free ^{125}I, cells and H_2O_2. Vectorial labeling of membrane proteins was extensive over a KI concentration range from 0.05 to 30.05 μM. The optimal KI concentration was 3.05 μM at a cell concentration ranging from 1 to 4 mg dry weight per ml of reaction mixture. Cells were not labeled in the absence of lactoperoxidase, whereas some vectorial iodination occurred in the absence of added H_2O_2. Iodinated components were resolved by SDS-polyacrylamide gel electrophoresis into at least 35 components ranging in molecular weights from 150,000 to 11,800 daltons. At least 8 principal surface proteins were observed, whereas numerous intermediate and minor proteins were noted. Vectorial labeling of several proteins by this technique indicates that the surface of *C. burnetii* cells is highly complex. An analysis of these surface components will help elucidate the nature of the interaction between *C. burnetii*, the host cell, and the immunologic apparatus of the infected host.

In collaboration with Dr. Kindmark, we have initiated studies on soluble antigens of *C. burnetii* which interact with plasma proteins. Recently we have shown that *C. burnetii* releases a soluble component which forms a circulating immune complex in the sera of experimentally infected guinea pigs. The appearance of a soluble antigen in the sera paralleled the anti-complementary activity and the inability of sera to lyse sheep red blood cells. The immune complex was very stable as evidenced by resistance to dissociation by heating at 56°C for 30 min, but heating in the presence of 3.5M iodine released an antigen from the immune complex. The circulating soluble antigen was shown to form a line of identity with a phenol-extracted soluble component by employing a modified immunoelectrophoretic technique. Establishment of the sensivitity and specificity of the method for the detection of circulating antigen may lead to a rapid diagnostic test in human acute and chronic infections.

Various immunological parameters were investigated in C57BL/10ScN (endotoxic non-responder) mice following i.p. administration of formalin-inactivated *C. burnetii* whole cells. Spleen cells explanted from mice pretreated with *C. burnetii* whole cells displayed a 20-fold increase in blastogenic activity as determined by increased ^{3}H-Tdr uptake. Pretreatment of mice with whole cells, however, suppressed the normal mitogenic response of spleen cells to ConA, PHA, and PWM. Suppression of the mitogenic response by whole cells was time and dose dependent. However, mice pretreated with whole cells demonstrated heightened resistance to infection with *Listeria monocytogenes*. Thus, nonviable *C. burnetii* whole cells are capable of suppressing host immune responsiveness to lymphocyte transformation by various mitogens while promoting a nonspecific resistance to heterologous challenge. The nature of the cellular and subcellular components responsible for the noted biological effects are currently under investigation.

The ELISA test for soluble antigens and antibodies is being investigated in collaboration with Dr. L. Thomas. The object of the collaboration is the characterization immunogenic components of C. burnetii vaccines. Several antigenic preparations, prepared by different extraction methods and chemical treatments, have been assayed by the ELISA test. Antigens are being selected for use in the detection of hybridoma antibody in the study of surface reactive determinants.

The future course of this project will be continued in three areas as specified below: (1) C. burnetii will be fractionated into cell wall and soluble components. The protective antigens will be localized and separated from toxic factors, and the efficacy of each component will be compared to the whole cell vaccine. (2) Membrane proteins, carbohydrates and other components will be compared in various strains of rickettsiae. Extracellular proteins and glycoproteins will be identified by ^{125}I-labeling patterns and two dimensional chromatography profiles. Carbohydrates will be identified by chemical assay, radiolabeling with sugars and specific staining. (3) Early detection of antigens and antibodies will be attempted by employing homogenous antigens and monospecific antisera. Monospecific antisera will be prepared by employing hybridoma techniques. Antibody classes and subclasses induced by various homogenous antigens will be analyzed during immunization as compared to infection carried out in mice, rabbits, and guinea pigs. Human sera will be evaluated as described for the animal models. The ELISA assay to detect specific phase antigens will be investigated.

Publications:

Dasch, G. A., Samms, J. R., and Williams, J. C.: Partial purification and characterization of the major species-specific protein antigens of Rickettsia typhi and Rickettsia prowazekii identified by rocket immunoelectrophoresis. Infect. Immun. 31: 276-288, 1981.

Williams, J. C., Peacock, M. G., and McCaul, T. F.: Immunological and biological characterization of Coxiella burnetii, phases I and II, separated from host components. Infect. Immun. 32: 840-851, 1981.

In press:

McCaul, T. F. and Williams, J. C.: Developmental cycle of Coxiella burnetii: Structure and morphogenesis of vegetative and sporogenic differentiation. J. Bact.

Williams, J. C. and Cantrell, J. L.: Biological and immunological properties of *Coxiella burnetii* vaccines in C57BL/10ScN endotoxin non-responder mice. *Infect. Immun.*

Williams, J. C., Peacock, M. G., and Kindmark, C. O.: Detection of *Coxiella burnetii* soluble antigens by immunoelectrophoresis: Demonstration of antigen in the sera of guinea pigs during experimental Q fever. In Burgdorfer, W. and Anacker, R. L. (Eds.): *Rickettsiae and Rickettsial Diseases*. New York, Academic Press.

Williams, J. C.: Iodination of *Coxiella burnetii* cells: Optimal conditions for the iodination of exposed surface proteins. In Burgdorfer, W. and Anacker, R. L. (Eds.): *Rickettsiae and Rickettsial Diseases*. New York, Academic Press.

Damrow, T. A., Cantrell, J. L., and Williams, J. C.: Modification of immune competence in mice by Q fever vaccine. In Burgdorfer, W. and Anacker, R. L. (Eds.): *Rickettsiae and Rickettsial Diseases*. New York, Academic Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00193-02 LMSF

PERIOD COVERED October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Gonococcal Surface Components: Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Swanson	Chief	LMSF NIAID
OTHER:	P. Hitchcock	Staff Fellow	LMSF NIAID
	R. Judd	Staff Fellow	LMSF NIAID
	O. Barrera	Microbiologist	LMSF NIAID

COOPERATING UNITS (if any)

None

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project concerns structure-function relationships of components on the surfaces of Neisseria gonorrhoeae to understand the mediation of biological reactivities of these organisms related to pathogenesis of gonorrhea. Work during the current year has focused on structural comparisons of different outer membrane proteins, immunochemical properties of surface-exposed outer membrane proteins, and factors of gonococcal virulence in the chick embryo.

Project Description:

Studies during the past year have concentrated on selected structural, immunochemical, and biological properties of the dominant outer membrane proteins that are exposed on the surfaces of gonococci. The structural and immunochemical studies have been directed toward understanding properties of proteins I, II, and III which appear to be exposed on the organism's exterior. The roles of pili as well as protein I constituents on virulence of gonococci in the chicken embryo model have been investigated.

1. Structural studies (Judd and Swanson). ^{125}I-peptide mapping has been carried out on the above-noted proteins after their separation by SDS-PAGE and radioiodination. Chymotryptic digestion of these proteins yields radiolabeled peptides that are separable by two dimensional (electrophoretic and chromatographic) separations on thin layer sheets. These studies have shown the following:

(a) Protein I's (porin-like proteins) from different strains fall into two structural groups on the basis of their ^{125}I-peptide patterns. These groups correlate with the apparent subunit molecular weights (by SDS-PAGE) for the proteins. This grouping has been corroborated through study of gonococcal strains with P.I different from those previously investigated. A significant degree of apparent structural homology is found among all P.I's when the ^{125}I-peptides are obtained after radioiodination of the whole, denatured protein subunits and subsequent peptide analysis.

(b) Although two protein I's of differing apparent subunit MW may have similar overall ^{125}I-peptide maps, the surface-exposed iodinatable peptides of the same two proteins may differ remarkably.

(c) Protein II constituents among different strains or in different colony forms of a single strain may differ in MW, but their overall ^{125}I-peptide patterns are fairly similar to one another. However, consistent differences are found for the heat-modifiable P.II's with different subunit MWs from different strains or within a single gonococcal culture. These differences are even more striking if surface-exposed radioiodinated peptides are compared.

(d) The protein III's from all strains and all intrastrain variants are virtually identical by ^{125}I-peptide mapping. Seeming identity for P.III's is also found when surface-exposed, iodinatable residues are compared.

(e) Slightly different ^{125}I-peptide maps are obtained for P.I's depending on whether whole cells or other membrane vesicles are radioiodinated for determining which surface-exposed moieties are liable to radiolabeling.

2. Immunochemical studies (Swanson and Barrera). Two different approaches have been used for study of the gonococcal antigens which are

recognized by sera raised in rabbits inoculated with whole gonococci. In the first, detergent solubilized gonococci were subjected to crossed immunoelectrophoresis (CIE) against antisera raised against homologous or heterologous strains and colonial phenotypes. This technique was combined with radioiodination of whole bacteria to trace the surface-exposed iodinated constituents of the gonococcal surface. In the second approach, radioimmunoprecipitation (RIP) was carried out on radioiodinated gonococci which were incubated with antisera either before or after lysis in detergent. Exposure to antiserum prior to lysis was used to define which radioiodinated outer membrane constituents were "seen" by IgG antibodies. Incubation of a detergent lysate should reveal immunochemical characteristics of both those portions of outer membrane proteins which are "buried" within the hydrophobic matrix of the membrane and those portions which are surface-exposed. The following results have been obtained:

(a) Both protein I and protein II are antigenic and immunogenic, and both have surface-sites available for interaction with IgG molecules of rabbit serum.

(b) Protein III does not seem to participate in RIP reactions upon incubation with antiserum either before or after lysis with detergent except that it is immunoprecipitated by antibodies directed against protein I (with which P.III forms a polymer, in situ).

(c) Antigenic differences among protein I's of different subunit MWs are demonstrable by RIP if gonococcus + antiserum incubation precedes lysis; addition of antiserum following detergent lysis suggests rather broad antigenic cross-reactivities among all protein I subunit MW forms.

(d) The presence or absence of protein II's (one or more) does not appear to interfere with combination of antibodies with exposed portions of protein I, in situ.

(e) Protein II's are antigenically quite distinct when different subunit MW forms of these proteins are compared by RIP. Clearly, more than one antigenic form of P.II can be found on individual organisms through RIP analysis.

3. Pathogenicity and virulence of gonococci in a chicken host (Hitchcock and Swanson). The chick embryo model has been used previously to study gonococcal virulence, but the results of those experiments were contradictory to some extent. A reexamination of the characteristics of gonococci that influence virulence of these organims inoculated intravenously into 10- to 11-d-old chick embryos has revealed the following:

(a) Finding significant differences between piliated and non-piliated gonococci (so-called "virulent" and "avirulent" colony types, respectively) is inconsistent; demonstrating such difference depends on such technical parameters as choice of suspending vehicle (for inoculation), cooling embryos prior to incubation or just prior to inoculation with gonococci.

(b) Differences in virulence are consistently found among gonococcal strains studied; these differences appear to correlate with the protein I constituent of a given gonococcal preparation. The degree or magnitude of differences among these different strains exceeds the differences between piliated and non-piliated gonococci.

Also, the use of oviduct explants from hens cultivated *in vitro* has been investigated. Preliminary experiments suggest that chicken oviducts respond to *in vitro* gonococcal infection analogous to that for human fallopian tube explants. This contrasts with lack of reactivities for other non-human species' oviducts as demonstrated by other workers. Co-infection of chicken oviducts with *Mycoplasma gallisepticum* and gonococci leads to enhanced survival of both the mycoplasma and the gonococci (as compared with single infections with each organism) and exaggerated damage of the oviduct epithelium.

Publications:

Swanson, J.: Gonococcal growth inhibition and hydrolysis of outer membrane proteins by alpha-chymotrypsin. In Danielsson, D. and Normark, S. (Eds.): *Gentics and Immunobiology of Pathogenic Neisseria*. University of Umea, Sweden, 1980, pp. 19-24.

Blake, M. S., Gotschlich, E. C., and Swanson, J.: The effect of proteolytic enzymes on the outer membrane proteins of *Neisseria gonorrhoeae*. *Infect. Immun*. 33: 212-222, 1981.

In press:

Swanson, J. and Mayer, L. W.: Biology of *Neisseria gonorrhoeae*. In Holmes, K. K., Mardh, P-A., Sparling, P. F., and Wiesner, P. J. (Eds.): *Sexually Transmitted Diseases*. New York, McGraw-Hill.

Swanson, J.: Surface-exposed protein antigens of the gonococcal outer membrane. *Infect. Immun*.

Swanson, J.: Gonococcal surface proteins. Proceedings of International Symposium on Bacterial Vaccines, September 15-18, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00194-02 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Molecular Genetics of Neisseria gonorrhoeae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. W. Mayer	Expert (Microbiology)	LMSF NIAID

COOPERATING UNITS (if any) Dr. M. Tam, Hutchinson Cancer Center, Seattle, WN

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Laboratory of Microbial Structure and Function, Hamilton, MT 59840

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2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The emphasis of this project is to use molecular genetics, including recombinant DNA techniques, to understand the pathogenesis of gonorrhea. Since the outer surface of the gonococcus has an important role in its ability to cause disease, we are currently studying the genetic control of expression of different molecules in the gonococcal outer membrane. We are attempting to isolate the genes for two major outer membrane proteins, to determine the DNA sequence of these genes and thus predict the primary structures and functions of these proteins. The mechanism of phase transition by which gonococci can alter the composition of their outer membrane is also being studied.

PHS-6040
(Rev. 10-76)

Project Description:

The main thrust of this project is understanding the control mechanisms which mediate the expression of the outer membrane proteins of the gonococcus. We are cloning the genes for the major membrane proteins (or protein I) as well as genes for some minor (protein II) species some of which are the opacity-associated proteins. Recent studies on the serological cross reactivities and non-cross reactivities of protein IIs have caused us to expand the scope of our cloning to include all four of the different prototype strains as classified by the molecular weight of their protein Is.

Using preparative scale electrophoresis, we have purified the protein I and two protein II species from the prototype strains. Only one protein II has been isolated from one strain. These proteins are isolated from purified outer membranes, and since they are prepared by acrylamide gel electrophoresis, they naturally show a single band on SDS-PAGE. These proteins are being used to immunize animals to prepare monospecific antisera which we will use to screen our gene banks for the antigens of interest. In cooperation with a group at the Hutchinson Cancer Center in Seattle, we are also obtaining monoclonal hybridoma antibody to these proteins. All these antisera still have to be utilized in control experiments before they can be used to identify _Escherichia coli_ clones expressing the proteins I or II of the gonococcus.

The gene banks we previously created do not seem to react with antisera raised against the gonococcal antigens. There are two factors which affect this: (1) the antisera are not very reactive; whole gonococci were used as immunogenes in some cases, and (2) the gene banks were constructed using high copy number plasmids as vectors. The outer membrane proteins are numerically abundant (about 10^5 molecules per cell). It has been shown in _E. coli_ that the gene dosage affect of having 20 copies of a gene, as in a high copy plasmid, makes is impossible to clone outer membrane protein genes because over production of this scale is a lethal event for the host cell. This makes outer membrane protein genes some of the most difficult to clone.

Since we have no information about the efficiency of the expression of gonococcal genes in _E. coli_, we feel the best approach is to generate a series of gene banks using different copy number vectors. By using pDF 41 (a single copy F derivative), pHC 75-58 (2-3 copy cosmid), and so forth as well as charon phage vectors, we hope to find the balance between poor expression and copy number.

Although the cloning of gonococcal genes in _E. coli_ is very exciting from a basic science standpoint, their expression in gonococci is the most interesting because of its relationship to the pathogenicity of the organism. Unfortunately all our current information indicates it will be very difficult to return genes into the gonococcus which have been cloned in _E. coli_. This suggests a need to develop a cloning system of all gonococcal components. We are trying to genetically combine the naturally occurring R-plasmids of gonococci with another transposon carrying a different resistance. Our choice of transposon is very limited. The transposons which carry tetracycline, kanamycin, sulfonamide, or many other antibiotic resistances cannot be used as these resistances have not yet occurred (or been observed to naturally occur) in gonococci. Even resistance to silver ions can't be used since silver nitrate prophylaxis is a wide practice. Tn-501 seems like a good candidate though as it carries resistance only to mercury ions. Although this transposon has not beey very well characterized, we feel it will be useful as a genetic element to construct a cloning system in gonococci as well as a tool to probe the gonococcal chromosome in a more conventional genetics manner.

Publications:

Mayer, L. W.: Colony opacity changes in N. gonorrhoeae. In Danielsson, D. and Normark, S. (Eds.): Genetics and Immunobiology of Pathogenic Neisseria. University of Umea, Sweden, 1980, pp. 135-137.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00195-02 LMSF

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Molecular Aspects of Mycoplasma-Eukaryote Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alan Liss	Expert (Molecular Biology and Bacteriology)	LMSF NIAID

COOPERATING UNITS (if any) Dr. J. Kloepper, Plant Pathology Department, Univ of California, Berkeley; Dr. K.-E. Johansson, Uppsala Univ, Sweden; Dr. A. Wieslander, Lund Univ, Sweden.

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TOTAL MANYEARS: 1.7	PROFESSIONAL: 1.0	OTHER: 0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-term goal of this project is to determine how the cell wall-less mycoplasmas interact with their eukaryotic hosts. Current emphasis involves the elucidation of the roles of viruses and plasmids in the determination of surface characteristics of mycoplasmal cells. Extra-chromosomal DNA's have been identified, using agarose gel electrophoresis, in several mycoplasma, acholeplasma and spiroplasma strains. Assignment of specific plasmids as intra-cellular forms of previously described viruses is underway. Several Acholeplasma laidlawii strains can be stimulated to release viruses after treatment with mitomycin C. Using spiroplasma strains, similar virus induction has been unsuccessful. Spiroplasma cells, when infected with group 1 virus, can survive suboptimal conditions better than uninfected spiroplasmas. Similarly infected A. laidlawii strains show no selective advantage. Whole cell polypeptide profiles, using polyacrylamide gel electrophoresis, reveal few if any differences between uninfected and chronically infected mycoplasmas. Continued work using the enveloped plasmaviruses is being carried out in an attempt to learn more about cell membrane involvement in the virus infection cycle.

Project Description:

Current work involves identification of the contributions from plasmids and/or viruses to surface properties of the cell wall-less mycoplasmas. In most studies Acholeplasma laidlawii is used because of its rapid growth and relatively non-fastidious growth requirements. Although apparently non-pathogenic itself, A. laidlawii is a viable model system for studies of cell surface phenomena shared by pathogenic mycoplasma species. Certain experiments involving virus carrier-state use the spiral, motile, cell wall-less prokaryotes, the spiroplasmas. These are naturally infected with a variety of viruses and often have several extra-chromosomal DNA's.

1. As noted preliminarily in last year's report, I have been able to stimulate the release of group 1 mycoplasma-virus from several A. laidlawii strains by treatment with mitomycin C. This does not appear to be an "induction" in the sense of eubacterial systems. Rather it appears that mitomycin C, at a concentration of 2.5 µg/ml, causes an imbalance in the normal (non-productive) intra-cellular virus vs host chromosome relationship. The result of this imbalance is the release of observable plaque-forming units. To further identify the nature of the imbalance, I have found that under mitomycin C concentrations that stop host DNA synthesis (i.e., host stops incorporation of labeled thymidine into DNA) virus replication can be demonstrated. Key to this study was the selection of a new virus indicator strain that was, itself, insensitive to mitomycin C concentrations used in previous studies.

Preliminary studies comparing properties of virus-carrying and "cured" A. laidlawii strains reveal no obvious differences in whole cell or membrane polypeptide profiles as displayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A comparison of plasmid profiles is underway.

2. Spiroplasmas isolated from many natural sources can be shown to be infected by one or more groups of viruses. Using spiroplasma strain BC3, we (work done in collaboration with Ramona A. Heiland, a summer student) have found that infected BC3 cells survive condition of sub-optimal nutrient concentrations or osmolarity better than uninfected cells. Host BC3 cells have several plasmids. Attempts are underway to identify a particular plasmid as being the intracellular form of the virus known to infect BC3. Preliminary data show that virus-carrying BC3 cells lose their ability to release plaque-forming units after treatment with acridine orange. A concomitant loss of a plasmid, however, has not yet been confirmed.

3. As noted in last year's report, there is a close relationship in A. laidlawii's ability to propagate virus and its membrane lipid composition. In collaboration with two Swedish laboratories, it has been demonstrated that mutations leading to a change in host cell phenotype from virus sensitive to virus resistant also results in changes in lipid composition, both qualitative and quantitative, of the host cell membrane. To further investigate the role of membrane lipid composition on the virus life cycle, a plasmavirus is

being studied. Plasmaviruses are the only truly enveloped prokaryotic viruses with MVL2 being the prototype. Preliminary studies by me as well as others show that plasmaviruses are budding viruses, and they appear to be modified by propagation in certain *A. laidlawii* hosts. The most obvious result of such modification is changes in plating efficiencies when using a bank of standard indicator strains. Biological characterization of a plaque-purified isolate of MVL2 grown on three *A. laidlawii* hosts is underway. Each host has similar SDS-PAGE polypeptide arrays but different lipid compositions. Preliminary one-step growth studies reveal that MVL2 has different growth cycles on each host. In all cases, however, infected cells do not appear to lyse directly due to the virus infection. Evaluation of the adsorption of each virus on each host (nine combinations of viruses and hosts) show adsorption constants ranging from 0.6 to 1.0×10^{-10} cm^3 min^{-1}. These values are within the range published, by others, for other plasmaviruses. Once this set of plasmaviruses is characterized, functional analysis of the steps in the virus life cycle will be investigated after altering the composition of host membranes. Such manipulation of cell membrane composition is possible by altering the nutritional supplements given each strain of mycoplasma. These studies may be useful in understanding how viruses bud through membranes as well as what effect, if any, plasmaviruses might have on mycoplasma host surfaces.

Publications:

Steinick, L. E., Wieslander, A., Johansson, K.-E., and Liss, A.: Membrane composition and virus susceptibility of *Acholeplasma laidlawii*. *J. Bact*. 143: 1200-1207, 1980.

Liss, A. and Cole, R. M.: Spiroplasmavirus group 1: Isolation, growth, and properties. *Curr. Microbiol*. 5: 357-362, 1981.

In press:

Liss, A., Ackermann, H.-W., Mayer, L. W., and Zierdt, C. H.: Tailed phages of *Pseudomonas* and related bacteria. *Intervirology*.

Liss, A., and Cole, R. M.: Spiroplasmaviruses: Group 1 characteristics. *Rev. Infect. Dis*.

Liss, A.: Release of a group 1 mycoplasmavirus from *Acholeplasma laidlawii* after treatment with mitomycin C. *J. Virol*.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00196-02 LMSF

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Bordetella pertussis: Outer Membrane Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. S. Peppler Expert (Microbiology) LMSF NIAID

COOPERATING UNITS (if any) Dr. David Bemis, Univ of Tennessee, Knoxville; Dr. Gail Cassell, Univ of Alabama, Birmingham; and Dr. Charles Manclark, FDA, BoB, Bethesda, MD

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.0	0.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to define the biochemical composition of the major surface constituents, especially the "agglutinogens," of Bordetella pertussis and their relationship to the pathogenicity and epidemiology of whooping cough. Isogenic clones of virulent and avirulent pertussis organisms are compared for differences in SDS-PAGE profiles after extrinsic labeling by ^{125}Iodine. Radio-immune precipitates of solubilized antigen-antibody complexes are used to identify virulent-associated bands which can be further characterized by peptide-mapping. These bands and/or their associated antigens are isolated for raising specific hyperimmune antisera and for screening hybridoma antibody. The specific antibodies so raised are then used for screening chemically-induced mutants altered in production of virulence-associated antigens. Analysis of specific mutants can define the genetics of virulence in B. pertussis for further understanding of pathological and immunological mechanisms of whooping cough.

Project Description:

The purpose of this project is to define the structure and function of outer membrane components which contribute to virulence in Bordetella pertussis. Principal candidates under investigation are fimbrial hemagglutinin (FHA), pertussigen (Pgn), lipopolysaccharide (LPS) and the serotype antigens (agglutinogens). As described by this laboratory, wild type B. pertussis organisms produce domed and hemolytic (Dm^+Hy^+) colonies on Bordet-Gengou agar (BGA) and possess all of the outer membrane components cited above. Stable phenotypic variants which produce flat, non-hemolytic (Dm^-Hy^-) colonies on BGA do not possess these outer membrane components (except LPS). A technique was developed for isolating isogenic pairs of Dm^+Hy^+ and Dm^-Hy^- organisms for the study of virulence-associated outer membrane components. This technique has been applied successfully to 13/13 strains tested. The Dm^-Hy^- variants have been further characterized as showing loss of adenylate cyclase activity, and their frequency within a population of Dm^+Hy^+ has been determined to be between 10^{-5} and 10^{-6}. Fatty acid methyl ester profiles obtained by gas liquid chromatography, however, appear similar when organisms of different phenotypes are grown on the same medium.

Dm^+Hy^+ and Dm^-Hy^- organisms extrinsically labeled with ^{125}I show markedly different SDS-PAGE autoradiographic patterns. This difference has been analyzed by a radioimmune precipitate assay (RIP) using antibody against FHA, Pgn, and agglutinogen 1. Autoradiograms of radioactivity-labeled antigens separated by SDS-PAGE confirm the specificity of the antibodies for the Dm^+Hy^+ phenotype but also reveal cross-specificities among antibodies previously shown to be different by double diffusion analysis. Antisera are currently being raised in mice by the method of Bronne-Shanbury and Dolby to prepare specific anti-agglutinogen reagents for resolving these conflicts. A co-agglutination assay using protein A-bearing staphylococci has been developed to monitor the immune IgG titers in the mice. This method has yielded clearer endpoints than bacterial agglutination and its use of protein A is consistent with the method of the RIP assay to which it can be correlated.

Peptide mapping has been used to identify relatedness of surface-labeled proteins within the SDS-PAGE profile of a single strain/phenotype, among strain/phenotypes, and between Dm^+Hy^+ organisms and their partially purified surface components. A high degree of similarity exists in the peptide maps of major outer membrane proteins of not only phenotypes or strains of B. pertussis but also among the species B. parapertussis and B. bronchiseptica. To date the band(s) on SDS-PAGE which corresponds to FHA or Pgn has not been determined by peptide mapping, although these experiments have just begun. The maps of two dominantly radioactive Dm^+Hy^+-specific SDS-PAGE bands from extrinsically ^{125}I-labeled whole cells are distinct from FHA or Pgn, suggesting a different class of surface proteins, perhaps the agglutinogens.

In addition to phenotypic variants with altered outer membrane proteins, *B. pertussis* also gives rise to LPS mutants (Nakase and Kasuga, 1953). I am currently selecting naturally occurring "rough" variants from our prototype strains to describe the frequency at which this variation can be detected and to define the biochemical difference between the "rough" LPS and LPS from an isogenic "smooth" phenotype from the same strain.

The requirement of absorbing *B. pertussis* antisera with other species of *Bordetellae* has led me to observe a phenotypic variation in *B. bronchiseptica* similar to that of *B. pertussis*. On BGA, Dm^{+}Hy^{++} *B. bronchiseptica* is non-flagellated whereas the isogenic Dm^{-}Hy^{-} phenotype is flagellated and motile. Organisms extrinsically labled with ^{125}I show phenotype-specific SDS-PAGE autoradiographic patterns. The patterns have a molecular weight distribution similar to the patterns seen in *B. pertussis* phenotypes. The nature of phenotype-specific antigens in *B. bronchiseptica* and their cross-reactions with *B. pertussis* antisera are being studied in collaboration with Dr. D. Bemis, University of Tennessee, Knoxville. A preliminary test showed no cross-reactivity for a *B. bronchiseptica* pilus preparation with *B. pertussis* FHA.

The respective virulence of the two *B. bronchiseptica* phenotypes is presently unknown. However, in collaboration with Dr. G. Cassell, University of Alabama, Birmingham, a rat model will be used to determine the ID$_{50}$ of the Dm^{+}Hy^{++}, Dm^{-}Hy^{-} organisms. Specific fluorescent anti-phenotype antisera developed in this laboratory will be used to determine phenotype *in vivo* during the course of infection.

A collaborative project has been established with Dr. C. R. Manclark at the FDA Bureau of Biologics, Bethesda, to determine the nature and specificity of the human antibody response in laboratory-acquired *B. pertussis* infection. The levels of IgG, IgA and IgM directed against FHA, Pgn, and agglutinogen 1 have been determined by ELISA for one series of pre- and post-infection sera and show distinct sero-conversion in all classes of antibody to agglutinogen 1 and FHA but only a weak response to Pgn. The combined information from three individual cases should give insight into fundamental questions concerning the epidemiology and serology of whooping cough.

Information gained from this project and from its collaborative studies will complement our understanding of the relationships between ultrastructure of the *Bordetellae* and their pathogenic function. It is hoped that this will provide a more solid genetic and biochemical basis for development of improved vaccines and serodiagnostics.

Publications:

Craven, D. E., Peppler, M. S., Frasch, C. E., Mocca, L. F., McGrath, P. P., and Washington, G.: Adherence of isolates of *Neisseria*

meningitidis from patients and carriers to human buccal epithelial cells. J. Infect. Dis. 142: 556-568, 1980.

Craven, D. E., Peppler, M. S., Frasch, C. E., Mocca, L. F., and McGrath, P. P.: The role of Neisseria meningitidis surface structures in the adherence of case and carrier isolates. In Danielsson, D. and Normark S. (Eds.): Genetics and Immunobiology of Pathogenic Neisseria. University of Umea, Sweden, 1980, pp. 189-193.

In press:

Frasch, C. E., Peppler, M. S., Cate, T. R., and Zahradnik, J. M.: Immunogenicity and clinical evaluation of Group B Neisseria meningitidis outer membrane protein vaccines. Proceedings of International Symposium on Bacterial Vaccines, September 15-18, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00215-01 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Action of Beta-lactam Antibiotics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. G. Barbour	Senior Staff Fellow	NIAID LMSF

COOPERATING UNITS (if any)

Dr. Charles B. Smith, Div Infectious Diseases, Univ of Utah Medical Center, Salt Lake City

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In this project I am attempting to further characterize the molecular targets of beta-lactam antibiotics in bacteria and to follow the events that occur in a bacterial cell after penicillin has bound to these targets. In pursuit of this I have studied the actions of beta-lactam antibiotics on Neisseria gonorrhoeae, Chlamydia trachomatis, and Borrelia hermsi during the preceding year. In addition to this main project, I have also examined strains of Staphylococcus aureus that were associated with cases of Toxic-Shock Syndrome (TSS) to determine whether these TSS strains differ in any way from other strains of S. aureus.

PHS-6040
(Rev. 10-76)

Mechanisms of action of beta-lactam antibiotics

During the past year I studied the actions of beta-lactam antibiotics on 3 types of bacteria, each of which demonstrated unique responses to penicillin and related compounds. The species studied were *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Borrelia hermsi*. Identification of the molecular targets of beta-lactam antibiotics in these 3 species should provide further insight into peptidoglycan synthesis and cell division of bacteria.

Investigations of the 3 penicillin-binding proteins (PBPs) located in the cytoplasmic membrane fraction of *N. gonorrhoeae* revealed that PBPs 1 and 2 bound less penicillin in penicillin-resistant strains than in penicillin-susceptile strains. One beta-lactam antibiotic in particular, mecillinam, bound to only one PBP (PBP 2) of *N. gonorrhoeae*, yet mecillinam inhibited cell division completely at a concentration which bound 50% of available PBP 2. In contrast to PBPs 1 and 2, PBP 3 does not appear to be a critical target of penicillin or other beta-lactam antibiotics.

The obligate intracellular parasite *C. trachomatis* also has 3 PBPs which are saturated by penicillin at nanomolar concentrations. In collaboration with Dr. H. Caldwell of LMSF, I found that penicillin at these low concentrations is also effective in stopping both division of the vegetative reticulate bodies and conversion of reticulate bodies to the infectious elementary bodies of *C. trachomatis* in cell culture. *C. trachomatis*' unusual cell wall does not contain detectable muramic acid, a constituent of the peptidoglycan of all other eubacteria.

The third organism, *B. hermsi*, is a pathogenic spirochete that can be cultured *in vitro*. Work performed in collaboration with Drs. H. Stoenner and W. Todd of RML has shown that *B. hermsi* has 5 PBPs and undergoes a unique morphologic change in the presence of the minimal inhibitory concentration of penicillin.

Pathogenesis of Toxic-Shock Syndrome

Toxic-shock syndrome (TSS) is a multi-system disease caused by *Staphylococcus aureus*. Although the major features of the disease would appear to be toxin-mediated, the pathogenesis of TSS is not yet understood. The reasons why TSS has so greatly increased in incidence in the U.S. during the last two years are also not known.

To identify possible pathogenic mechanisms and to find markers useful for both epidemiologic and genetic studies, I compared a group of TSS-associated strains of *S. aureus* with a group of vaginal isolates of *S. aureus* from asymptomatic women. These specimens were obtained through collaboration with Dr. C. B. Smith of the University of Utah Medical Center. This study showed the TSS strains differed from non-TSS strains in more commonly producing an extracellular protein with molecular weight of 22,000 and isoelectric point of 7.0, being arsenate resistant, and proteolysing hemoglobin. On the other hand, TSS strains were unexpectedly less hemolytic in agar media and less toxic for laboratory animals than non-TSS strains. In current studies, I am trying to identify which, if any, of the numerous toxins and other extracellular proteins excreted by S. aureus stimulate detectable, specific antibody elevations in TSS patients during the course of illness and convalescence.

Publications:

Barbour, A. G., Mayer, L. W., and Spratt, B. G.: Mecillinam resistance in Escherichia coli: Dissociation of growth inhibition and morphologic change. J. Infect. Dis 143: 114-121, 1981.

Barbour, A. G.: Properties of penicillin-binding proteins in Neisseria gonorrhoeae. Antimicrob. Agents Chemother. 19: 316-322, 1981.

In press:

Barbour, A. G.: Vaginal isolates of Staphylococcus aureus associated with toxic shock syndrome. Infect. Immun.

Kehrberg, M. W., Latham, R. H., Haslam, B. T., Hightower, A., Tanner, M., Jacobson, J. A., Barbour, A. G., Noble, V., and Smith, C. B.: Risk factors for staphylococcal toxic-shock syndrome. Am. J. Epidemiol.

Barbour, A. G. and Spruance, S. L.: Colorado tick fever. In Hoeprich, P. D. (Ed.): Infectious Diseases, 3rd edition. Hagerston, Harper and Row.

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00216-01 LMSF

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunochemistry of Chlamydial Surface Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. D. Caldwell Expert (Microbiology) LMSF NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chlamydia trachomatis has been recently implicated as the causative agent for several disease syndromes whose etiologics were previously unknown: nongonococcal urethritis, epididymitis, acute proctitis, cervicitis, salpingitis, neonatal inclusion conjunctivitis and pneumonitis. The frequencies of these infections indicate a clear need for improved diagnostic methods and disease prevention. This project is concerned with those two long-term objectives by first defining the immunologic properties of components on the surfaces of C. trachomatis organisms in an attempt to understand the antigenic properties of these immunological accessible macromolecules. Current work is focused on the major outer membrane protein (MOMP) as an antigen that may have application in the future development in both the immunodiagnosis and immunoprophylaxis of C. trachomatis infections. The MOMPs of several C. trachomatis strains are being purified by preparatory sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and characterized both antigenically and structurally.

PHS-6040
(Rev. 10-76)

Project Description:

During the current year the project aim of defining antigenic and structural properties of the MOMP of *C. trachomatis* organisms has concentrated on purification of these proteins from several *C. trachomatis* strains, producing antisera against them, evaluating the specificities of these sera, and their uses in assessing the biological functions of MOMPs.

MOMPs from the *C. trachomatis* serotypes (B, D, G, H, and L2) and the *C. psittaci* Meningopneumonitis (Mn) strain were isolated by preparatory SDS-PAGE. The subunit molecular weights of the isolated MOMPs varied among the serotypes and were: B (39.5 Kilodaltons), D (40.5K), G (38K), H (44.5K), L2 (39.5K), and Mn (40K). Hyperimmune rabbit antisera were prepared against these isolated SDS-polypeptide complexes and their specificities determined by both rocket immunoelectrophoresis with the soluble polypeptide as antigen and by micro-immunofluorescence (micro-IF) using whole elementary bodies from each of the *C. trachomatis* serotypes and four *C. psittaci* strains. Immunological cross reactions were observed by rocket immunoelectrophoresis among each of the isolated MOMPs from the five *C. trachomatis* serotypes but not with the MOMP of the *C. psittaci* Mn strain. Anti-MOMP sera were also species-specific by micro-IF, however, distinct serological differences occurred with these antisera when they were reacted with whole chlamydiae. Anti-MOMP antibody titers were much greater for the homologous antiserum-elementary body reaction. Anti-MOMP sera of the B, D, G, and L2 serotypes were strongly cross-reactive with all *C. trachomatis* serotypes except the A, C, H, I, and J serotypes. In contrast, anti-MOMP serum of the H serotype had a high antibody titer to its homologous elementary body antigen, but this serum was only weakly cross-reactive with the 14 other *C. trachomatis* serotypes. *C. trachomatis* isolates were separated into two distinct serogroups on the basis of their serologic reactions with anti-MOMP sera. Interestingly, hyperimmune antiserum prepared against viable L2 elementary bodies reacted with an identical specificity as antiserum against the L2 (39.5K) MOMP suggesting that the *C. trachomatis* MOMP may be a serodominant antigen. Taken together, these findings indicate that the MOMP of *C. trachomatis* is antigenically complex and that antigenic heterogenicity exists particularly among the surface-exposed portions of the macromolecule. In attempts to ascertain these possible differences, comparative structural analysis of the isolated polypeptides was performed by chymotryptic ^{125}I-peptide mapping. Peptide maps of the MOMPs of each of the *C. trachomatis* serotypes were distinctly different from those of the *C. psittaci* Mn strain. Conversely, peptide maps of each of the *C. trachomatis* MOMPs studied were very similar. Unique peptidic units were observed for individual *C. trachomatis* MOMPs with the most peptide microheterogeneity occurring with the MOMP of the H serotype. Thus, these preliminary structural analyses of the proteins correlated extrememly well with the observed immunological findings.

The isolated IgG fraction from anti-MOMP sera neutralized viable *C. trachomatis* organisms *in vitro* with virtually the same specificity observed by micro-IF. Antibodies against the L2 (39.5K) MOMP prevented attachment of ^{14}C-radiolabeled L2 organisms. These findings suggest that antibodies directed against the MOMP of *C. trachomatis* may be protective in preventing infection. Animal model studies are planned to evaluate the role of this antigen(s) as vaccines *in vivo*.

Publications:

Caldwell, H. D., Kromhout, J., and Schachter, J.: Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infect. Immun.* 31: 1161-1176, 1981.

Laboratory of Persistent Viral Diseases
Rocky Mountain Laboratories
Hamilton, Montana
1981 Annual Report
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Annual Report
Laboratory of Persistent Viral Diseases
Rocky Mountain Laboratories
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1980 to September 30, 1981

RESEARCH HIGHLIGHTS

Monoclonal antibodies specific for xenotropic and MCF murine leukemia viruses have been produced. These antibodies are able to distinguish different substrains of xenotropic MuLV and should permit more accurate analysis of these endogenous virus loci. Many of these antibodies were obtained during graft vs. host reactions *in vivo*, and this suggests a possible role for xenotropic MuLV in this syndrome.

Role of antiviral antibody in control of virus expression in leukemia cells in vivo has been analyzed by passive transfer of monoclonal anti-Friend virus antibodies. Only IgG_{2a} anti-gp70 antibodies could restrict virus release *in vivo* in spite of the fact that all other antibodies tested could react with leukemia cell surfaces.

A Friend virus-specific T lymphocyte proliferation assay indicated that the Rfv-1 (H-2D) gene influenced the kinetics of development of FV-specific T lymphocytes following virus inoculation. This kinetic difference might account for the influence of the Rfv-1 gene on recovery from leukemia.

A non-virion protein induced by Aleutian disease virus has been found in ADV-infected cells. Only mink with progressive immune complex disease appear to have a strong antibody response to this protein. Monoclonal antibodies to ADV virion protein do not recognize this nonstructural protein.

Two mouse strains have been found to be resistant to intraperitoneal inoculation of rabies virus . Comparison of these mouse strains with susceptible strains should allow genetic and functional analysis of mechanisms of resistance to rabies.

Female Protein (FP), the hamster acute phase protein, showed a paradoxical response to inflammatory stimuli. In contrast to C-reactive protein in man, serum FP levels *decreased* in female hamsters following acute inflammation.

ADMINISTRATIVE REPORT

Dr. Leonard Evans joined the LPVD as a Senior Staff Fellow. He was previously at the Department of Molecular Biology, University of California at Berkeley.

Annual Report
Laboratory of Persistent Viral Diseases
Rocky Mountain Laboratories
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1980 to September 30, 1981

HONORS AND AWARDS

Editorial Boards of Journals:

Dr. B. Chesebro

Associate Editor - Journal of Immunology
- Journal of the National Cancer Institute
- Proceedings of the National Academy of Sciences

Dr. D. L. Lodmell

Associate Editor - Journal of Immunology

Professional Posts:

Dr. B. Chesebro - Adjunct Professor - Department of Microbiology, Montana State University, Bozeman

Dr. M. E. Bloom - Faculty Affiliate, Department of Microbiology, University of Montana, Missoula

Dr. J. E. Coe - Faculty Affiliate, Department of Microbiology, University of Montana, Missoula

Dr. D. L. Lodmell - Faculty Affiliate, Department of Microbiology, University of Montana, Missoula

Invited Lectures and Participation in Meetings and Symposia:

Dr. B. Chesebro - Invited Speaker, Friend Cell Symposium, Seillac, France

Dr. J. Coe - Invited Speaker, Rush Medical School, Chicago

- Invited Speaker, New York Academy of Sciences, New York

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NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00072-10 LPVD

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Host Defense Mechanisms in Viral Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. L. Lodmell Scientist Director LPVD NIAID

COOPERATING UNITS (if any)

Dr. A. Dwight Lopes, The Wistar Institute, Philadelphia, Pennsylvania;
Dr. Bill Quinn, Montana Veterinary Diagnostic Laboratory, Bozeman, Montana

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to delineate the mechanisms of host resistance to viral diseases. The principal model for these studies involves investigating the pathogenesis and neuroimmunology of rabies virus-infected mice, and ascertaining the mechanisms(s) by which paralyzed mice abort their central nervous system infections and recover from disease. A sensitive and specific assay has been developed for determining the importance of cytolytic antibody in recovery from infection. It was found that mice which recovered from disease had high cytolytic antibody titers in brain, serum and cerebrospinal fluid. Genetic studies with inbred strains of mice have shown that A/WySn and A.SW/Sn mice were 100% susceptible to intraperitoneally injected rabies street virus, whereas SJL/J and CBA/J mice were 100% resistant. There was no evidence that resistance was H-2 linked or that sex-linked factor was of importance. Resistance was not manifested until mice were at least four weeks old. Studies with hybrid F_1 generations indicated that resistance was dominant.

PHS-6040
(Rev. 10-76)

Project Description:

This project is designed to elucidate the mechanism(s) by which paralyzed rabies virus-infected mice abort their central nervous system affliction and recover from disease. Recovery from rabies virus infections has been reported in man as well as various animals, but the process by which recovery occurs is unknown.

Influence of cell type and virus upon lysis of rabies virus-infected cells by antibody and complement. To determine the importance of cytolytic antibody in recovery from rabies virus infections, a sensitive and specific assay for measuring this antibody was developed. Mouse neuroblastoma (MNB), BHK-21/S13 or CER cells were infected with either challenge virus standard (CVS), ERA or Flury high egg passage (HEP) strains of virus. It was determined that MNB cells infected for 24 hr with the ERA strain of virus was the combination of choice. Cells used at this time were >80% positive for both cytoplasmic and membrane fluorescence. Furthermore, antisera from mice injected with either HEP Flury, CVS, ERA or street viruses readily lysed ERA-infected MNB cells in the presence of rabbit complement. MNB cells infected with the CVS strain also were suitable targets. It was, however, difficult to infect MNB cells with the Flury HEP strain of virus and even when 90% of the cells were positive for membrane viral antigen, cytolytic antibody titers were consistently 8- to 16-fold lower than on CVS or ERA-infected MNB cells. It also was determined that CER cells were unsatisfactory for the demonstration of cytolytic antibody.

Two unusual findings were demonstrated in this study. First, some cell populations which were >80% positive for rabies viral membrane antigens were poorly lysed with antirabies antibody in the presence of complement (MNB-HEP, CER-ERA and CER-HEP). Second, cell populations were detected which expressed cytoplasmic antigen, but lacked membrane antigen(s) (CER-CVS, BHK-21/S13-CVS). The lack of membrane antigen(s) on virus-infected cells may be of importance in persistent infections. Dogs, skunks and bats have been shown to excrete virus in their saliva for extended periods of time following nonfatal infections (the carrier state).

Susceptibility of mice to rabies street virus. The possibility that inbred strains of mice may vary in their susceptibility to rabies street virus was investigated.

It was determined that A/WySnJ (H-$2^{a/a}$) and A.SW/SnJ (H-$2^{s/s}$) mice were 100% susceptible to intraperitoneally injected rabies street virus, whereas SJL/J (H-$2^{s/s}$) and CBA/J (H-$2^{k/k}$) mice were 100% resistant. DBA/2J (H-$2^{d/d}$) mice were 80% resistant, whereas C57BL/10ScN (H-$2^{b/b}$) and B10.A (H-$2^{a/a}$) were 50% resistant. Strains less than 50% resistant, but not 100% susceptible were A/J (H-$2^{a/a}$), C57BL/6 (H-$2^{b/b}$) and BALB.B (H-$2^{b/b}$). The resistance does not appear to be H-2 linked as shown by the results with SJL/J and A.SW/Sn mice. There was no evidence that a sex-linked factor conferred this resistance which was not manifested until after the fourth week of age. SJL/J mice not only were 100% resistant to street virus, but most (94%) did not develop signs of illness throughout the time frame of the experiments; 30% of the CBA/J did become sick, but all recovered. Preliminary studies with hybrid F_1 generations indicated that resistance was dominant. A/WySn X SJL/J or CBA/J F_1 were 100% resistant, as were B10.A X SJL/J, CBA/J or DBA/2J F_1. Interestingly, the F_1 of two susceptible strains (B10.A X A/WySn) also were 100% resistant, suggesting that each parental strain possessed a unique set of alleles at different foci which in the heterozygous F_1 generation interreacted to confer resistance. Backcross experiments (C57BL/10ScN X SJL/J)F_1 X C57BL/10ScN tentatively suggested resistance was controlled by one gene.

Antibody determinations of the different strains of mice which aborted infections indicated that both cytolytic and neutralizing antibody titers were high in brains, cerebrospinal fluids and sera of these mice. In contrast, SJL mice which usually did not become ill had minimal antibody titers.

Preliminary data suggested that there was no major difference among the strains in their capacity to produce interferon after injection with either rabies virus or the nonspecific interferon inducer, poly ICLC.

Kinetic studies suggested there was no difference in the ability of rabies virus to replicate in the brains of resistant or susceptible mice after intracerebral inoculation. There also was no difference in the $ICLD_{50}$ between A/WySnJ and SJL/J mice.

These studies promise to provide insight into the elucidation of possible mechanisms of recovery from a virus infection of the central nervous system (CNS) which almost always ends in death. This work also should enhance our understanding of the immune capabilities within the CNS, and how we may possibly manipulate this system to enhance recovery from rabies, as well as other viral infections of the CNS.

The future course of this project will focus on understanding the relative roles of humoral and cell-mediated immune responses in abortive rabies virus infections. Major emphasis will be placed on studies with mice which are either 100% susceptible or resistant to intraperitoneally injected street virus. Genetic studies with parents, F_1, F_2 and backcross mice also will be pursued in the hope that immunological or other mechanisms of resistance will become apparent in these strains, which then can be utilized in our understanding of the abortive infection phenomenon.

Publications:

In press

Lodmell, D. L., Cent, R. R., Jr., Pusateri, A. M., and Ewalt, L. C.: Nonspecific inhibition of encephalomyocarditis virus (EMCV) replication by a nonphagocytic adherent cell from athymic nude mice sensitized with nonviable *Mycobacterium tuberculosis*. *Proceedings Third International Workshop on Nude Mice*.

Lodmell, D. L. and Portis, J. L.: Immunologic and genetic aspects of Aleutian disease. In Gershwin, M. E. and Merchant, B. (Eds.): *Immunologic Defects in Laboratory Animals*.

Lodmell, D. L., Arai, Y. T., and Ewalt, L. C.: Influence of cell type and virus upon lysis of rabies virus-infected cells by antibody and complement. *Arch. Virol*.

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PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immunity and Immunopathology in Virus Related Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	F. J. Waxman	Senior Staff Fellow	LPVD NIAID

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	2.0	1.7

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been studying inflammation and immunity in the Syrian hamster. A sex limited serum protein of females called Female Protein (FP) was shown to be functionally similar to other homologous acute phase proteins , C-reactive protein (CRP) and serum amyloid P component (SAP). FP binds complement when reacted with poly-L-arginine (similar to CRP) and is found in hamster amyloid deposits (similar to SAP). The acute phase response consists of a decrease in the normally high levels of serum FP in female hamsters in contrast to the typical increase serum levels of SAP and CRP seen in other mammals and also FP in serum of male hamsters. In regard to hamster immune response, the humoral and cellular immune response of the hamster spleen to soluble protein antigens (but not particulate antigen) was found deficient. A population of normal spleen cells (nylon wool nonadherent) added to sensitized lymph node cells, markedly suppressed the antigen specific proliferation. Numerous IgG$_1$ and IgG$_2$ antibody forming precursors were detectable in spleen cells after in vitro culture of sensitized spleen cells incubated in the absence of antigen. Many antibody forming cells were found in spleens of recipients reconstituted with sensitized lymph node cells.

PHS-6040
(Rev. 10-76)

Project Description:

Studies on hamster Female Protein, a paradoxical acute phase protein. Female protein (FP) is a significant (1.0 to 2.0 mg/ml) serum constituent in normal Syrian hamster females. This pentraxin is homologous to serum amyloid P component (SAP) and C-reactive protein (CRP) present in other mammals (including man). In contrast to FP, SAP and CRP are normally present in low serum concentration but do achieve high serum levels (up to 1 mg/ml) under various pathological conditions and therefore have been designated acute phase proteins. The functional significance of acute phase proteins is unknown, however, the high serum levels of FP normally present in female hamsters may provide a suitable model for evaluating the advantage/ disadvantage of this acute phase protein response. In contrast to SAP and CRP, serum levels of FP fall rapidly (within 24 to 48 hr) after injection of turpentine or croton oil into female hamsters. Studies in progress with I^{125} labeled FP will attempt to explain this apparent consumption or deposition of FP in the acute phase, however, the normal biological half life of FP has been found to be rather short (10 to 12 hr) even in the normal hamster. Male hamsters normally have low (<.025 mg/ml) serum levels of FP, which rapidly (24 to 48 hr) increases in serum concentration after noxious stimulation in a typical acute phase response. Similar to CRP, FP will bind to phosphorylcholine in a Ca^{++} dependent fashion. The functional relevance of FP to CRP has been further extended by the finding that FP will bind complement when it reacts with poly L arginine, although the complement binding is less efficient than CRP. The functional relevance of FP to SAP is now more apparent, in that FP has been found to be present in hamster amyloid (liver, spleen, kidney) as detected by specific immunofluorescence and amyloid tissue extracts. Amyloid disease has been induced in Syrian hamsters by Na caseinate injections and diethylstilbesterol treatment. The innate sex variation and potential hormonal manipulation of serum FP levels in this model should provide new insight into the influence of FP (and by inference SAP) on generation of amyloid.

Studies on the hamster spleen, an immunologically deficient organ. The inoculation of Syrian hamsters with hen egg albumin or bovine serum albumin in complete Freund's adjuvant induced the development of numerous antigen-specific IgG_1 and IgG_2 antibody-secreting cells in draining lymph nodes, but few in the spleen. This "splenic unresponsiveness" is remarkable when contrasted with the murine spleen. The antigen-induced proliferative response of hamster spleen cells was also lower in magnitude than the response of draining lymph node cells. This impaired splenic proliferative response was not due to a shift in culture kinetics, or in the optimal dose of antigen required to induce proliferation in vitro. The impaired antibody and proliferative responses of the spleen were apparently independent of the sensitizing dose of antigen, and the route of sensitization. The splenic deficit did not reflect an absolute impairment of spleen cells to proliferate, since some proliferation was detected when spleen cells were cultured at a high cell density in the presence of large amounts of antigen. Spleen and lymph node cells developed comparable proliferate responses when cultured with mitogen. In contrast to the results using soluble protein antigens, numerous antibody-secreting cells developed in the spleen following challenge with a particulate antigen. These data indicate that the hamster spleen is deficient in some, but not all, types of immune responses.

The impaired hamster splenic proliferative response was not enhanced by the depletion of adherent cells. However, the addition of nylon wool nonadherent spleen cells to sensitized draining lymph node cells markedly suppressed antigen-specific proliferation. This suppressor cell activity was found in the spleens of nonsensitized hamsters, and thus was antigen nonspecific. Nevertheless, suppression could be overcome by culturing cell mixtures in the presence of large quantities of antigen. Suppressor cell activity was radioresistant, and was not detected in lymphoid organs other than the spleen. Suppression was not due to a general impairment of the ability to proliferate, since mitogen-induced proliferation was not effected.

This splenic deficiency is apparently not due to a reduction in the number of antibody-forming cell precursors, since numerous IgG_1 and IgG_2 antibody-secreting cells appeared following *in vitro* culture of sensitized spleen cells incubated in the absence of antigen. A similar expansion of antibody-secreting cells did not occur in sensitized lymph node cell cultures incubated under the same conditions. The capacity of splenic antibody-forming cell precursors to develop into antibody-secreting cells upon adoptive transfer into lethally irradiated recipients was also evaluated. In contrast to results obtained *in vitro*, adoptively-transfered spleen cells failed to develop into antibody-secreting cells *in vivo*. However, the adoptive transfer of sensitized lymph node cells resulted in the appearance of numerous antibody-secreting cells in the recipient's spleen. This suggests that the splenic humoral immune response regulatory mechanism is sensitive to irradiation and thus may differ from the radioresistant suppressor cell apparently involved in regulation of antigen-specific proliferation. Collectively, these data suggest that the hamster splenic immune response is regulated at the level of the development of antigen-reactive cells into effector cells.

Future goals include the following: 1) Characterization of the cellular populations involved in regulating the hamster splenic response to soluble protein antigens in contrast to particulate antigens. Different suppressor cell subpopulations may regulate humoral and cellular immune responsiveness. Efforts to develop monoclonal reagents with specificity for hamster lymphoid cell populations are in progress. 2) Characterization of regulatory macromolecules involved in regulating the hamster splenic response to soluble protein antigens. It will be of interest to characterize the molecular nature of the pulative suppressive signal which is antigen-nonspecific, but yet whose activity can be overcome by a sufficient quantity of antigen.

Publications:

Coe, J. E.: Immunoglobulins: Hamster, guinea pig, rabbit, and chicken. In Altman, P. L. and Katz, D. D. (Eds.): Inbred Genetically Defined Strains of Laboratory Animals, Part 2. Federation of American Societies for Experimental Biology, 1979, pp. 470-471.

Murphy, F. A., Bell, J. F., Bauer, S. P., Gardner, J. J., Moore, G. J., and Coe, J. E.: Experimental chronic rabies in the cat. Lab Invest. 43: 231-241, 1980.

Waxman, F. J., Perryman, L. E., Hinrichs, D. J., and Coe, J. E.: Genetic resistance to the induction of experimental allergic encephalomyelitis in Lewis rats. I. Genetic analysis of an apparent mutant strain with phenotypic resistance to experimental allergic encephalomyelitis. J. Exp. Med. 153: 61-74, 1981.

Coe, J. E.: Comparative immunology of old world hamsters -- Cricetinae In Streilein, J. W., Hart, D. A., Stein-Streilein, J., Duncan, W. R., and Billingham, R. E. (Eds.): Hamster Immune Responses in Infectious and Oncologic Diseases. New York, Plenum Publishing Corp., 1981, pp. 95-101.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00074-09 LPVD

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Host Defense Mechanisms in Chronic Viral and Neoplastic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	M. W. Cloyd	Senior Staff Fellow	LPVD NIAID
	L. Evans	Senior Staff Fellow	LPVD NIAID

COOPERATING UNITS (if any) Dr. J. Hartley, Laboratory of Viral Diseases, NIAID, Bethesda, MD; Dr. W. Rowe, Laboratory of Viral Diseases, NIAID, Bethesda, MD; Dr. C. Kozak, Laboratory of Viral Diseases, NIAID, Bethesda, MD

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.7	4.0	2.7

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main goal of this project is the understanding of host defense mechanisms involved in recovery from virus-induced leukemia in mice. Individual mouse genes have been identified which appear to influence T cell-mediated or humoral virus-specific responses and the interactions of these mechanisms have been studied. Monoclonal antibodies specific for various viral proteins have also been selected and the biological roles of antibodies of different specificities and Ig classes are being studied both in vivo and in vitro.

PHS-6040
(Rev. 10-76)

Project Description:

Mechanisms of recovery from FV leukemia. Spontaneous recovery from Friend virus (FV)-induced erythroleukemia occurs in some mouse strains. We have identified three mouse genes, Rfv-1, Rfv-2 and Rfv-3 which influence the recovery process, and we are currently studying the mechanisms of their effects on this disease. Rfv-1 and Rfv-2 are both associated with the H-2 complex. Rfv-1 is in the H-2D subregion, and Rfv-2 has not been accurately mapped. Rfv-1 appears to operate through its expression in nonleukemic cells of the spleen and bone marrow. Our most recent data using an FV-specific T lymphocyte proliferation assay indicates that Rfv-1 controls the rate of development of virus-specific T lymphocytes, whereas Rfv-2 may operate as a dominant immune response gene controlling the presence of this response. Thus, $H-2^{a/a}$ mice fail to develop this response. In contrast, $H-2^{a/b}$ mice develop the response between 16 and 30 days postinoculation, but $H-2^{b/b}$ mice become positive 6 to 10 days after virus injection. H-2 recombinant mice indicate that this difference is controlled by the H-2D subregion.

The actual elimination of leukemic spleen cells appears to be mediated by virus-specific cytotoxic T lymphocytes (CTL). This has been demonstrated by adoptive transfer of splenic T lymphocytes from immune mice of various Rfv-1 genotypes. Furthermore, using erythroleukemia cell lines derived from mice with H-2 recombinations, we have shown that anti-FV CTL can kill leukemic target cells via H-2-restricted recognition of $H-2D^b$ or $H-2D^d$ antigens plus FV antigens. This finding supports the conclusions of previous adoptive transfer experiments suggesting that lack of recovery in mice which have only the $H-2D^d$ allele ($H-2^{a/a}$ and $H-2^{d/d}$ mice) is not due to inability of anti-FV CTL to function in H-2 restricted lysis of cells with these genotypes.

The Rfv-3 gene interacts with Rfv-1 and Rfv-2 genes in the process of recovery from leukemia. The $Rfv-3^{r/s}$ genotype is necessary but not sufficient for recovery. However, this genotype is sufficient for developing a strong humoral immune response to FV, even in the presence of persistent malignant splenomegaly. This response is capable of eliminating viremia and drastically reducing the virus release by leukemic spleen cells. These effects can be accelerated by passive transfer of immune serum from $Rfv-3^{r/s}$ mice. Most recently we have studied the role of antiviral antibodies in recovery from FV leukemia and viremia by passive transfer of monoclonal antibodies derived in this laboratory. Cytotoxic IgG_{2a} anti-gp70 antibodies caused a decrease in spleen cell infectious centers analogous to that seen spontaneously in $Rfv-3^{r/s}$ mice. Cytotoxic IgM anti-gp70 and IgG_{2b} and IgG_{2a} anti-p15 were unable to cause this decrease in spite of their ability to lyse these cells in vitro in the presence of complement. The mechanism of this decrease of infectious centers is under current investigation.

Role of cell surface gp70 in viral interference. We have recently discovered a clone of FV-induced erythroleukemia cells with mutations resulting in abnormal viral protein expression and processing. The core precursor protein $pr65^{\underline{gag}}$ is incorporated into virions in an unprocessed form. Perhaps subsequent to this defect, these virions have a 90% reduction in envelope glycoprotein (gp70) in spite of an abundance of this protein on the cell surface. We have now found that these mutant cells can be easily reinfected with nondefective FV whereas a related leukemia cell clone releasing normal virions shows interference to superinfection. These results indicate that mere presence of cell surface gp70 does not result in resistance to superinfection. One possible interpretation of our data is that newly released normal virions mediate interference by receptor blockade, whereas the envelope-deficient virions released by the mutant clone cannot block the receptors due to lack of sufficient envelope glycoprotein.

Use of hybridoma antibodies and oligonucleotide mapping as probes for viral gene structure and virus recombinations. A series of monoclonal antibodies specific for Friend-MCF viral gp70's has been derived. These antibodies have been used as a panel to look for heterogeneity among F-MCF viruses cloned from individual leukemic mice. Substantial heterogeneity was demonstrated even in viruses obtained from the same individual leukemic spleen. Leukemogenicity of these isolates is currently being tested to see which portions of the genome are necessary for neoplastic transformation.

Several cloned ecotropic Friend helper viruses have been used to induce leukemia in newborn mice. Hybridoma probes and oligonucleotide mapping indicate significant differences exist among these strains. Recombinant (F-MCF) viruses derived from these helpers are now being isolated to determine whether different types of recombinants are formed with different helpers.

Similarly, the role of heterogeneity among isolates of spleen focus-forming virus (SFFV) is being studied. The anemia and polycythemia inducing strains of SFFV were previously shown to differ markedly. An SFFV isolate derived from a leukemia (YA97) induced by the polycythemia strain lacks two oligonucleotides found in the "parental" SFFV strain. Work is in progress to determine whether these changes are due to deletions or base changes.

Publications:

Collins, J. K., Britt, W. J., and Chesebro, B.: Cytotoxic T lymphocyte recognition of gp70 on Friend virus-induced erythroleukemia cell clones. J. Immunol. 125: 1318-1324, 1980.

Collins, J. K. and Chesebro, B.: Synthesis, processing, and cell surface expression of Friend and xenotropic murine leukemia virus gp70 antigens on Friend virus induced erythroleukemia cell clones. J. Immunol. 125: 1325-1331, 1980.

Britt, W. J. and Chesebro, B.: Recovery from Friend virus leukemia is determined by the H-2 genotype of nonleukemic cells of the spleen and bone marrow. In Skamene, E.,Kongshavn, P. A. L., and Landy, M. (Eds.): Genetic Control of Natural Resistance to Infection and Malignancy. New York, Academic Press, 1980, pp. 329-335.

Britt, W. J. and Chesebro, B.: H-2D (Rfv-1) gene influence on recovery from Friend virus leukemia is mediated by nonleukemic cells of the spleen and bone marrow. J. Exp. Med. 152: 1795-1804, 1980.

Collins, J. K. and Chesebro, B.: Replication-defective Friend murine leukemia virus particles containing uncleaved gag polyproteins and decreased levels of envelope glycoprotein. J. Virol. 37: 161-170, 1981.

Chesebro, B.: The influence of major histocompatibility complex (H-2) on oncornavirus-induced neoplasia in mice. In Kaiser, H. E. (Ed.): Neoplasms - Comparative Pathology of Growth in Animals, Plants and man. Baltimore, Williams and Wilkins, 1981, pp. 475-482.

Chesebro, B., Wehrly, K., Cloyd, M., Britt, W., Portis, J., Collins, J., and Nishio, J.: Characterization of mouse monoclonal antibodies specific for Friend murine leukemia virus-induced erythroleukemia cells: Friend specific and FMR-specific antigens. Virology 112: 131-144, 1981.

In press:

Britt, W. J., Collins, J. K., and Chesebro, B.: A new cell surface gp70 related to Friend mink cell-focus-inducing virus is expressed on Friend virus-induced erythroleukemic spleen cells following elimination of ecotropic Friend virus gp70 in Rfv-$3^{r/s}$ mice. J. Exp. Med.

Chesebro, B., Collins, J. K., Wehrly, K., Nishio, J., and Cloyd, M.: Expression of cell surface Friend virus gp70 does not block reinfection by ecotropic murine leukemia viruses. Virology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00085-04 LPVD

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biology of Aleutian Disease Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. E. Bloom	Medical Officer (Res)	LPVD NIAID
OTHER:	R. E. Race	Veterinary Officer	LPVD NIAID
	L. Mayer	Expert (Microbiologist)	LMSF NIAID

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NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to study Aleutian disease virus (ADV), a non-defective parvovirus, that causes a persistent infection of mink associated with immune complex disease. Studies on ADV DNA have defined a double stranded (ds) ADV DNA intermediate that should prove suitable for restriction enzyme analysis of the viral genome and molecular cloning. Studies on ADV proteins have revealed that a 71,000 dalton nonstructural protein (p71) as well as the two major structural proteins (MW 85,000 (p85) and 75,000 (p75)) were immunoprecipitated by sera from mink with progressive AD. p71 was not immunoprecipitated by heterologous antisera, monoclonal antibodies prepared against purified ADV, or some sera from mink with nonprogressive disease. The role of this protein in the genesis of immune complexes is under study.

PHS-6040
(Rev. 10-76)

Project Description:

Studies on intracellular ADV proteins. I previously showed ADV had two major structural proteins with MW's of 85,000 (p85) and 75,000 (p75) daltons. Lysates of [^{35}S]methionine-labeled infected Crandall feline kidney (CRFK) cells were prepared and immunoprecipitated with sera of AD affected mink and formalin inactivated S. aureus (SA). These samples contained p85 and p75 as well as a 71,000 (p71) protein not found in purified virions. p71 was not present in uninfected CRFK cells. Since ADV induced the same three proteins in mink cells, it seemed likely that p71 was a virus-specified nonstructural protein. All three polypeptides were evident after a 10 min "pulse" label and were still present after 24 hr of "chase" and thus no precursor-product relationship could be inferred. Furthermore, all three proteins could first be detected between 24 to 48 hr after infection and maximum synthesis corresponded with appearance of viral fluorescent antigen. When a panel of sera was reacted with aliquots of a single lysate of [^{35}S]methionine-labeled infected cells, heterologous antisera prepared against purified ADV did not contain detectable anti-p71 but precipitated p85 and p75, thus suggesting major antigenic differences between p71 and the two structural polypeptides. Furthermore, although all sera from mink with evidence of progressive AD (hypergammaglobulinemia) reacted with p85, p75 and p71, some sera from animals with antiviral antibody but no evidence of progressive disease did not contain detectable antibody to the nonstructural p71. This latter group of mink (nonAleutian mink inoculated with low virulence Pullman strain ADV) also shows the greatest heterogeneity of clinical response to ADV and we are investigating a possible correlation of anti-p71 with progressive disease.

Studies on ADV DNA. The purpose of this work is to develop reagents suitable for analyzing ADV at the DNA level. In collaboration with Drs. John Janick and Claude Garon at LBV, NIAID, NIH, additional work has been done with ADV virion DNA (vDNA). Using the Kleinschmidt technique for electron microscopy of DNA molecules, we have confirmed a MW of $\sim 1.4 \times 10^6$ daltons ($\sim$4.4 kilobases) for vDNA. The self priming 3' terminus (hairpin) has been utilized to generate a double stranded (ds) DNA molecule from vDNA and analysis of this species on agarose gels with restriction endonucleases has begun. This approach has been limited by the difficulty in preparing large quantities of vDNA. Consequently, I have isolated a ds ADV DNA present in infected cells. By analogy with other parvoviruses, this would be the replicative form (RF). The method is a recently described modification of the Hirt extraction technique for isolating replicative forms of viral DNA's and relies on the fact that parvovirus RF's contain unit length dimers covalently attached at the 3' end (a structure similar to that generated by Dr. Janick) that rapidly reanneal ("snap back") following heat denaturation. It has been relatively easy to isolate quantities of ds DNA suitable for analysis on agarose gels without prior radiolabeling and I have begun analyzing this DNA at RML in collaboration with Dr. Leonard Mayer, LMSF, RML. To date, using digestion with restriction enzymes and the Southern technique for DNA "blot transfer hybridization" with ^{32}P-labeled ADV vDNA as a probe, we have shown that this 4.6 kbp ds DNA hybridizes to vDNA and contains sites for several restriction enzymes. Ultimately, I plan to develop a detailed restriction map of the DNA and clone as much of the genome as possible. The availability of these reagents will allow us 1) to define the cellular location and the state of the viral genome in persistently infected tissues using methods of in situ DNA-DNA hybridization, and 2) to compare genomes of various strains of ADV that differ in virulence. Studies similar to these have recently been accomplished in two other nondefective parvovirus systems - minute virus of mouse and feline panleukopenia virus-canine parvovirus.

The results of the work described above have provided us with the information and methodology to undertake a number of future projects. Plans for future studies include:

1) Role of p71 in Aleutian disease (AD). Sera of mink afflicted with AD contain antibody to the nonstructural p71. In collaboration with Dr. Race, I plan to determine whether p71 is a constituent of the immune complexes that deposit in renal glomeruli. Since heterologous antisera and monoclonals directed against purified ADV virions do not have anti-p71 activity, we are attempting to produce an anti-p71 by inoculating mice with CRFK cells infected with ADV. If any mouse sera contain anti-p71, we will then try to generate anti-p71 monoclonals.

An additional approach is to isolate immunoglobulins from glomeruli and assay these proteins for antibody activity against the viral proteins. This approach has been successful for lymphocytic choriomeningitis virus (LCMV).

2) Peptide mapping. The summed molecular weights of the ADV proteins exceeds the theoretical coding capacity of the genome. If the three major proteins (p85, p75 and p71) are translated from a single mRNA or a set of mRNA's processed from a single precursor RNA (as has been proposed for other parvoviruses), the peptide maps should reveal a great deal of homology. I plan to study the interrelationship of these polypeptides with mapping techniques. Preliminary studies using ADV extrinically labeled with ^{125}I are in progress, but ultimately similar studies on [^{35}S]methionine labeled proteins from immunoprecipitates will also be done. This latter technique will allow the comparison of the nonstructural p71 to p85 and p75.

3) Site of in vivo viral replication. At the peak of viral replication, ADV antigen has been identified by immunofluorescence in phagocytic cells of the lymphoreticular system, including Kuppfer cells of the liver. I plan to isolate Kupffer cells from infected mink liver and to examine these infected cells for evidence of viral specific protein synthesis using [^{35}S]methionine labeling and immunoprecipitation. I have adapted the technique for Kupffer cell isolation to mink and we are currently doing this study. If viral protein synthesis can be demonstrated in this population of cells, an attempt will be made to detect viral DNA from these cells using the same method we are using for CRFK cells. The sensitivity afforded by the Southern blot transfer should enable us to detect and analyze viral DNA.

4) Site of in vivo viral persistence. Aleutian mink infected with ADV have a persistent infection complete with viremia and progressive disease (hypergammaglobulinemia and renal lesions). Although nonAleutian mink may have a nonpersistent infection, particularly following inoculation with a low virulence strain of ADV, it has recently been shown that nonAleutian mink may also have persistent infection of lymphoid tissue and a persistent viremia without progressive disease. The site of persistent infection and the state of the viral genome in Aleutians and nonAleutians are unknown. Using probes for ADV DNA and protein, this problem should also be amenable to analysis. Evidence from other parvovirus systems (mentioned above) would suggest that a host cell restriction on viral replication might play a role.

Publications:

Bloom, M. E., Race, R. E., and Wolfinbarger, J. B.: Characterization of Aleutian disease virus as a parvovirus. J. Virol. 35: 836-843, 1980.

Bloom, M. E.: Slow virus disease. In Feigin, R. D., and Cherry, J. D. (Eds.): Textbook of Pediatric Infectious Diseases, Vol. II. Philadelphia, W. B. Saunders Co., 1981, pp. 1412-1426.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
OFFICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00086-04 LPVD

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immune Recognition of Viral Antigens in Persistent Viral Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	M. W. Cloyd	Senior Staff Fellow	LPVD NIAID

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TOTAL MANYEARS: 2.0
PROFESSIONAL: 1.0
OTHER: 1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main goal of this project is understanding the role of virus encoded cell surface proteins in murine leukemia and their possible role in the maintenance of normal hematopoetic cell homeostasis. We have concentrated our recent efforts on developing monoclonal antibodies specific for viral polypeptides of distinct classes of MuLV. Certain mouse strains undergoing adult graft-versus-host response induced by parent→F_1 adoptive transfer develop serum antibody to MuLV. We have developed a series of hybridomas from these mice which detect unique determinants of ecotropic, xenotropic and MCF MuLV envelope polypeptides. Of particular importance is a series of monoclonal antibodies reacting with group- or strain-specific xenotropic MuLV env determinants. The viral specificities of serum antibodies generated during GVHR was found to vary depending on the mouse strains used and the evidence suggests that antibody production may be influenced by endogenous proviral genes of the donor strain. We are currently using the monoclonal antibodies to define the strain and organ distribution of various MuLV env determinants in both high and low leukemia mouse strains.

Project Description:

Humoral immune response during GVHR. The transfer of parental spleen cells into an immunocompetent F_1 host initiates a complex set of immunologic interactions grouped on the general term graft versus host response (GVHR). Soon after cell transfer, antihost cytotoxic T lymphocytes are detected in the lymphoid organs of the recipient. It is generally agreed that this donor antihost response is responsible for many of the untoward effects of GVHR. Little, however, is known of the role of serum antibody. Antibodies to host alloantigens have not been readily demonstrable possibly because of the large antigenic "sink" available for absorption. A few reports of antibody deposition in the tissues during GVHR suggest that humoral factors may participate in lesion development. For example, acute epidermal necrolysis, a serious consequence of human GVHR, is associated with little lymphocytic infiltration but the deposition of Ig and C3 at the dermal-epidermal junction is observed in a majority of cases. In addition, glomerulonephritis seen in certain mouse strains during chronic GVHR is accompanied by the deposition of Ig and C3 in glomeruli.

During attempts to recover anti-H-2 antibodies from mice undergoing GVHR, we observed that cell fusion products from several mouse strains secreted antibody not to alloantigens but with specificity for murine leukemia viruses. This observation has led to a series of experiments designed to study the possible role of this antigen-antibody system in GVHR. In addition, because these antibodies were derived from mice without prior immunization with exogenous virus, this system provided an opportunity to study the expression of endogenous viral gene products with highly specific probes.

We have concentrated our efforts on two recipient F_1 strains, (B6 X D2)F_1 and (NFS X AKR)F_1 injected with parental cells. Both donors and recipients have been young mice from 4 to 5 weeks of age. Several significant findings have emerged from these studies. 1) The incidence of recovery of anti-MuLV hybridomas was dependent on the donor strain. Hybridomas were produced with high frequency from (B6 X D2)F_1 recipients of D2 but not B6 parental cells and from (NFS X AKR)F_1 recipients of AKR but not NFS parental cells. This observation correlated with the incidence of serum antibody in the recipient strains. One hundred percent of (B6 X D2)F_1 recipients of 60 X 10^6 D2 cells developed anti-MuLV serum antibody by the third week after transfer whereas antibody was not detectable at a 1:10 dilution in the reciprocal transfer. Similar observations were made in (NFS X AKR)F_1 recipients. 2) The viral specificity of hybridomas derived during GVHR was influenced by the donor/ recipient strain combination. In seven separate fusions of spleen cells from (B6 X D2) recipients of D2 spleen cells 10 to 16 days after transfer, 74% of tissue culture wells from the primary fusion products contained antibody to xenotropic MuLV. In contrast, 68% of the wells from fusions of (NFS X AKR) recipients of AKR spleen cells contained antibody specific for unique determinants of a subclass of MCF virus. Although studies are not complete, it appears that these differences in viral specificity are reflected in the serum antibody response.

Polypeptide specificities of GVHR hybridomas. Eighteen MuLV-specific hybridoma cell lines have been cloned and their viral polypeptide specificities analyzed by SDS PAGE analysis. All of the antibodies which precipitated a product resolved by PAGE analysis reacted with an env encoded polypeptide. Three types of specificities were detected: anti-gp70, anti-p15(E) and anti-gp90 (pr90). The latter reactivity was characterized by the resolution of a gp70-p15(E) disulfide linked complex under nonreducing conditions from ^{125}I-surface labeled virus infected cells and the preferential precipitation of pr90 (peptide bonded gp70-p15(E)) from cells biosynthetically labeled with ^{35}S-methionine. These gp90 (pr90) specific determinants have been observed previously with hybridomas to certain MCF viruses and appear to be conformational in nature.

Viral specificities of GVHR hybridomas. Viral specificities were studied by membrane immunofluorescence using a panel of cloned MuLV infected cells including 7 ecotropic, 10 xenotropic, 2 amphotropic and 14 MCF viruses. Four classes of viral specificities were detected: 1) MuLV group specific, reacting with most of the ecotropic, xenotropic and MCF viruses, 2) ecotropic specific, 3) xenotropic specific, and 4) MCF specific. Of particular interest were the monoclonal antibodies reactive with xenotropic MuLV. Within this group were two antibodies which detected different ubiquitous xenotropic determinants and five antibodies which reacted with two nonoverlapping subclasses of xenotropic MuLV. These are, to our knowledge, the first xenotropic MuLV-specific hybridomas that have been described.

The MCF specific antibodies derived from AKR→(NFS X AKR)F_1 reacted with a subclass of recombinant MCF viruses of which AKR 247 MCF was the prototype. The origin of these determinants is unclear for they were not detected on any of the ecotropic, xenotropic or amphotropic viruses in our panel. Thus, it is our preliminary impression that they were created by env gene recombination or are expressed on a minor subclass of xenotropic viruses not represented in our panel.

Use of xenotropic MuLV specific hybridomas to detect parentage of MCF viruses. MCF MuLV have been causally related to thymoma development in certain high leukemia strains of mice. These viruses are env gene recombinants between ecotropic MuLV and sequences related to xenotropic virus. It has not been possible to serologically type the xenotropic portion of these recombinant viruses due to lack of specific reagents. However, it is known from structural studies that xenotropic env products are quite heterogeneous. We are currently analyzing a number of MCF viruses with the xenotropic MuLV-specific antibodies. Preliminary results indicate that two types of xenotropic specific determinants are detected. 1) xenotropic group specificity expressed by the majority of MCF's tested, and 2) xenotropic strain specific determinants expressed by subclasses of MCF viruses. It is the general goal of this study to identify relatedness of MCF viruses to specific strains xenotropic MuLV in order to allow more precise sequence comparisons.

Studies on the anti-MuLV antibody response during GVHR. As mentioned, the induction of anti-MuLV antibody in GVHR was dependent on the donor strain. In addition, H-2 typing of several of the GVHR hybridomas indicated that they were of recipient origin. This suggested that recipients may be responding to antigens whose expression is influenced by genes of the donor. We are currently in the process of studying the genetics of this system in (B6 X D2)F_1 mice. Preliminary evidence suggests that the anti-xenotropic MuLV response of the recipient is influenced by no more than two genes of the D2 donor. Linkage studies are in progress to determine if these genes are associated with the known MuLV structural genes of this strain.

To complement these studies, we are currently attempting to detect possible mouse strain differences in the expression of specific xenotropic MuLV env determinants. This work may shed light on the polymorphism of this antigen system and may provide information on the role of these determinants in differentiation.

Publications:

Portis, J. L. and McAtee, F. J.: Dissociation of H-2 recognition by antibody and cytotoxic T cells of a cloned murine leukemia cell line. Immunogenetics 12: 101-115, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00199-02 LPVD

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunobiology of Aleutian Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. E. Race	Veterinary Officer	LPVD NIAID
OTHER:	B. Chesebro	Medical Officer (SR SURG)	LPVD NIAID
	M. E. Bloom	Medical Officer (Res)	LPVD NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, Montana 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to define immune mechanisms and viral characteristics important in the pathogenesis of Aleutian disease. A lymphocyte proliferative assay was utilized to find mink with advanced Aleutian disease (AD) capable of generating an in vitro T lymphocyte response to viral and specific protein antigens. The assay will allow further study of the relation of T lymphocyte function to viral persistence and hypergammaglobulinemia. Another aspect of the project utilizes monoclonal antibodies to study antigenic differences among ADV isolates. These reagents are also being used as probes to locate viral antigen(s) in tissues of infected mink. Antibodies with three predominant patterns of reactivity have been identified. 1) those that react with viral antigens extracted from tissues of infected mink, 2) those reacting with viral antigens purified from ADV infected cultures, and 3) those reacting with virus from either source. Characterization of the antibodies obtained, and generation of new specificities will continue.

Project Description:

Proliferative responses in AD infected mink. Aleutian disease (AD) is a naturally occurring parvovirus caused disease of mink in which persistent viremia and a vigorous but non-neutralizing antiviral host response occur simultaneously. Immune complexes consisting of viral antigen, antibody and complement result and have been demonstrated in the circulation and tissues of infected mink. Their occurrence relates to the predominant pathology including proliferative glomerulonephritis, vasculitis, system plasmacytosis and hypergammaglobulinemia. The simultaneous and prolonged occurrence of hypergammaglobulinemia and viral persistence suggest aberrant cellular immunity in AD. We are using a lymphocyte proliferative assay to study the cellular immune response of AD infected mink to viral and protein antigens and the "T" cell mitogen Concanavalin A (Con A). We found mink, regardless of genotype, capable of responding in vitro to viral antigens. They also responded to keyhole limpet hemocyanin (KLH), if they were sensitized to it prior to ADV infection. They also responded to Con A. They did not respond to a normal antigen or to specific antigens they were not sensitized to. Response to viral antigen was observed in mink with advanced Aleutian disease but not in mink early after infection or in those with nonprogressive infections. Proliferative response did not correlate well with antiviral antibody titer or serum gammaglobulin levels, nor did it correlate with any protective or antiviral effect. The cells responding to ADV antigen were contained within the nylon wool nonadherent population whereas optimum KLH and con A responses were obtained with unfractionated cell populations. Our findings indicate that "T" cell function is not necessarily abrogated in AD infected mink. Subtle aberrations in regulatory networks may, nevertheless, be important in regulating immunoglobulin production or influence viral persistence. Perhaps, cells that ordinarily limit expansion of antigen reactive cells are inactivated by specific viral mechanisms or overwhelmed by massive antigen presentation. Viral specific "T" cell expansion and concomitant plasma cell accumulation might then continue unabated. We will utilize this assay to look for such regulatory subsets and also examine the influence of immune complexes on viral specific cellular immune response.

Monoclonal antibody characterization of ADV isolates. Aleutian disease virus has been successfully adapted to in vitro tissue culture systems. However, virus maintained in culture through several passages has often lost or exhibited reduced infectivity for mink. We sought to define antigenic differences between cultured virus and tissue derived virus and among various ADV isolates by obtaining monoclonal antibodies with anti-Aleutian disease virus (ADV) specificities. Hybridomas were generated by fusing the spleens of mice immunized with gradient purified ADV and NS-1 mouse myeloma cells. Hybridomas were screened by several techniques including polyacrylamide gel electrophoresis (PAGE), immunofluorescence, radioimmunoassay and counterimmunoelectrophoresis. Antibodies with three predominant patterns of antiviral reactivity were obtained. 1) A group with specificity for viral antigens extracted from tissues of infected mink, 2) A group that recognizes viral antigens purified from infected tissue culture cells, and 3) A group that reacts with virus from either source. Thus, hybridoma antibodies that distinguish private as well as common antigens between the two major ADV sources have been obtained. These reagents will be used to further distinguish among viral isolates and as probes to locate viral antigen(s) in tissues of infected mink. Cells and tissues important in viral replication might be identified. *In situ* characterization of tissue bound immune complex should also be possible. Further characterization of the antibodies obtained and generation of monoclonal antibodies with new specificities will continue.

Publications:

None

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RESEARCH HIGHLIGHTS

The following sections summarize this year's activities in our research program and discuss their significance relative to the epidemiology and ecology of the disease agents under investigation. The results of some of these studies and earlier ones are embodied in 25 publications. An additional 28 papers are scheduled to be published in various scientific journals, books, or conference proceedings.

RICKETTSIOSES

Our collaborative studies have continued to provide us with field, microbiological and clinical materials from which we can obtain new information about rickettsiae and rickettsial diseases in this country and elsewhere. We continue to participate as a World Health Organization Centre for Rickettsial Reference and Research and are providing reference reagents and expertise, particularly for tickborne rickettsial diseases and Q fever, to various domestic and foreign health agencies.

The thrust of our research effort has continued toward goals stated in the last several annual reports. However, rickettsial surveys of ixodid ticks that bite man or are involved in the natural maintenance of *Rickettsia rickettsii* in various areas of the United States are winding down. We now believe that we have fairly comprehensive knowledge of the kinds of rickettsiae that systemically infect the more important species of man-biting ticks. This fund of information should form the basis for systematic comparative investigations of biochemical, genetic, and antigenic composition of nonpathogenic and pathogenic rickettsiae, as well as fundamental ecologic interrelationships that bear on focality of tickborne rickettsioses. Some of these studies are now underway. Specific observations follow.

Two major experimental studies included evaluation of dogs as sources for infecting ticks with *R. rickettsii* and other spotted fever-group agents, and the "interference" phenomenon as a possible means to limit and/or prevent distribution of infected ticks. Dogs inoculated with, or fed upon by ticks infected with *R. rickettsii*, *R. rhipicephali*, or *R. montana* were studied for occurrence of disease, presence of rickettsemia, efficiency as sources of infection for noninfected ticks, and serologic response. *R. rickettsii*-infected dogs developed rickettsemias lasting 4 to 7 days with concentrations of organisms sufficient to infect 0.8% of simultaneously feeding normal ticks. Only one of the two strains of *R. rickettsii* gave rise to illness. Dogs infected with *R. rhipicephali* or *R. montana* did not develop illness or rickettsemia. Contrary to suggestions in several recent reports, these findings are evidence that dogs probably are not important natural sources for infecting ticks with R. rickettsii. Additional experiments were conducted to substantiate "interference" between spotted fever group rickettsiae in the arthropod host. In agreement with preliminary observations reported last year, it was not possible to superinfect with R. rickettsii, larval ticks ovarially infected with either R. montana or R. rhipicephali. Likewise, D. marginatus infected with R. slovaca could not be superinfected with R. rickettsii. The mechanisms of interference are still unclear but may be related to the failure of secondarily acquired rickettsiae from adsorbing to the surface of already infected cells. Ultrastructural investigations are in progress to explore this possibility using persistently infected vole cells as an experimental model. Continued experimental investigations concerning "sexual transmission" of R. rickettsii in the arthropod host provided no evidence of rickettsial survival during maturation of spermatozoa beyond the spermatid stage. Mechanical transfer of rickettsiae in fluids and epithelial cells of the spermatophores of infected males may occur, but this mechanism does not appear to induce generalized infection of the female and her progeny. Ultrastructural analysis of rickettsiae in persistently infected vole cells by high voltage electron microscopy led to the detection of tube-like extensions from the outer cell membrane that appear to interact with the cytoskeletal network of host cells. The nature and function of these structures is being investigated. Collaborative studies with the University of Neuchâtel on the ecology of several kinds of rickettsiae in Ixodes ricinus and Dermacentor marginatus from Switzerland continue. Several native rodent species were examined for their potential as possible extra-arthropod reservoirs of these agents. Serologic surveys suggest that Apodemus, Clethrionomys, and Microtus spp. may serve that function for the Swiss agent. (Burgdorfer)

The 1980 studies of tickborne rickettsiae in California were completed. Two findings of major importance emerged from these investigations. The R. canada-like isolate was confirmed as being indistinguishable from the #2678 prototype recovered in Ontario in 1963 on basis of DNA base composition (determined by Dr. Anacker) and a variety of phenotypic properties. This is only the second strain of this species of rickettsia to be isolated. Our results and those of others suggest that R. canada is geographically widely distributed in its arthropod host, Haemaphysalis leporispalustris, and that this rickettsial species probably comprises a biotype distinct from both the typhus- and spotted-fever groups. Routine serologic testing of tick-associated illnesses by us over the years has provided no evidence that R. canada is a source of human infection, and extensive surveys in various areas of the United States have not revealed the presence of R. canada in man-biting ticks. Additional circumstantial evidence based on cross antibody absorption tests was obtained that the R. rickettsii-like, 364D strains transmitted by D. occidentalis are responsible for the spotted fever-like illnesses in coastal California. Since these organisms are biologically slightly different from classical strains of R. rickettsii, the subspecies name, Rickettsia rickettsii californica, is being proposed for these rickettsiae, 21 strains of which have been examined to date. Continued characterization of isolates frequently encountered in the vectors of Rocky Mountain spotted fever that form a serogroup previously referred to as 369C has revealed a typhus- as well as a spotted fever-group component. Although the spotted fever group serologic cross-reactivity is more pronounced, DNA base composition determined by Dr. Anacker showed the G + C content to be indistinguishable from that of the typhus group. However, the genome size, and DNA homology of 369C with other rickettsiae have not been determined. Because of the marked phenotypic differences of 369C from other rickettsiae, the new species name, *Rickettsia belli* (after Dr. E. J. Bell), is being proposed for this organism. We have isolated strains of this serogroup from most areas in the US that we have surveyed and from 8 species of ticks. Routine testing of sera from tick-associated illnesses has provided no evidence of an etiologic association with this organism. We have encountered dual infection of ticks with *R. belli* and *R. montana* or *R. rhipicephali* on three occasions and Burgdorfer was unable to show interference in attempts to superinfect with *R. rickettsii* ticks ovarially infected with 369C. Therefore, it is unlikely that *R. belli* is important in the ecology of *R. rickettsii*. (Philip, Casper)

A resurgence of Q fever has occurred in the United States in the last several years due in part to the use of sheep for experimental work in some major medical centers. A large outbreak recently occurred at the University of Colorado involving 65 of 107 employees. In collaboration with Dr. Meiklejohn, we have had the opportunity to thoroughly evaluate the use of the microagglutination and microimmunofluorescence tests for serodiagnosis of primary Q fever. This experience, along with an increasing number of chronic cases studied, has demonstrated the superiority of these newer serologic techniques over the widely used complement fixation tests. (Peacock)

Our studies with Legionnaires' disease have been curtailed and results of earlier work are being evaluated for publication. At least three cellular antigens of *Legionella pneumophila* are released into liquid yeast extract broth media over a 4-day period of growth including one which is a major cell surface component. Scanning electron microscopy at intervals during this period defined the morphologic changes associated with this process. Acridine orange was shown to be a useful stain for identifying those organisms in which vital functions are fully intact. Soluble and particulate antigens prepared from strains of *L. pneumophila* representing 6 serogroups were used in enzyme-linked immunosorbent assay (ELISA) and microagglutination (MA) tests, respectively, to examine serologic relationships to infection of guinea pigs. Patterns of reactivity in the two tests differed. ELISA was generally, but not always, more sensitive, particularly with early serum antibody. Specificity of the tests showed no consistent patterns of cross reaction. Sometimes MA measured group specific reactions better than ELISA; at other times the reverse was true. Considerable heterogenicity in cross reactions was evident among strains in each of several serogroups. This variability indicates need for more than one antigen or a polyvalent preparation in these test systems. Variations in test results also indicate that more than one kind of test are sometimes needed for optimal serologic measurement of antibody response to infection. (Peacock, Thomas)

SYSTEMATICS OF TICKS

As in years past, this project continues the systematic treatment including taxonomy, biology, ecology and colonization of parasitic acarina of medical importance. Ours is the only tick collection in the world that contains all of the described genera (20). Some 88% of all described species are reposited here. A particular honor this last year was the designation by the American Society of Parasitologists of the Rocky Mountain Laboratories tick collection as a national resource center for type and voucher specimens, specimen data and related literature.

The scanning electron microscope (SEM) continues to be a useful taxonomic tool. Work continues on sorting out the _Ornithdoros capensis_ complex. _O. muesebecki_ has been redescribed, and SEM photos to illustrate this species are completed. A study of the _Ixodes_ (_Afrixodes_) _oldi_ complex from Africa has resulted in the description of five new species as well as characterization of the three known species. Studies of rhipicephalid ticks also continue. During the year an extensive review of the _Rhipicephalus simus_ complex indicates a likelihood of three, or possibly four, species comprising this group. Much of our material comes from collaborative studies. A large collection of ticks from Iraq, an area where information is scanty, was identified. Several species new to the RML collection were obtained from this material. Large collections of ticks from Mali are being sorted out in collaboration with several scientists from Texas A & M University.

In May, a Datapoint 1800 Dispersed Processor with diskette and printer were installed at RML. We are now being instructed in use of this equipment that will be used to enter new tick records and other data into the Smithsonian-NIH data file.

Work in collaboration with the British Mueum of Natural History on recataloguing the Nuttall collection was completed, and an 1800-page manuscript was prepared. Because of budgetary restrictions, it now appears that the catalogue will have to be converted to microfiche which unfortunately will restrict accessibility to this information. (Clifford, Keirans)

ARTHROPOD-BORNE VIRUSES

In the project to determine occurrence and ecology of tickborne disease agents, 19 viral, one spiroplasmal, and 21 rickettsial isolates were recovered from pools of some 3,000 ticks originating in 10 states and two foreign countries. Three previously described, one new, and two as yet unidentified viruses were obtained. Included were 5 isolates of Yaquina Head, an Orbivirus of the Kemerovo serogroup customarily found in migratory-bird ticks, the protoype of which was identified as a source of human infection in Central Europe and Asia. Also recovered from the same tick species, _Ixodes uriae_, were 3 isolates of an unidentified virus of the Sakhalin group (Bunyaviridae). Presence of these 2 viruses in the North Pacific points to a transoceanic continuum of tickborne viruses among migratory seabirds. Other viruses isolated this year include Quaranfil from _Argas arboreus_ in Egypt, Colorado tick fever virus, Cascade virus from D. occidentalis in Oregon, and an unidentified agent from A. cooleyi collected at Mono Lake in California. A manuscript describing Cascade, a previously undescribed virus, has been submitted for publication.

In collaboration with French and Japanese investigators, we examined the identities of certain viruses obtained from the circumglobal Ornithodoros capensis complex of argasid ticks that harbor its own spectrum of viruses ecologically equivalent to Kemerovo-Sakhalin-Uukuniemi serogroups found in ixodid ticks. Soldado, an argasid tick virus of the Hughes serogroup, was recently reported by French workers to exist as a number of antigenically distinct subtypes. Our isolates of this virus from various geographic areas were supplied to these investigators and specific antisera are being prepared for comparison with other Soldado-like strains. Hirota virus, a newly described Arenavirus from Japan, was shown to be serologically indistinguishable from Midway virus, an agent earlier reported by us from O. capensis ticks in the Hawaiian chain.

Last year, in collaboration with the Laboratory of Infectious Diseases, we reported the discovery of a new and unique group of spiroplasms from man-biting ixodid ticks in Oregon. We isolated an additional strain this year from I. pacificus. In view of the fact that several cases of erythema chronicum migrans from the West Coast were possibly associated with the bite of this species of tick, a possible etiologic relationship with this spiroplasm should be considered.

Rickettsiae recovered this year included another isolate of the unclassified Tillamook (Quarry)-like agent from I. pacificus in Oregon and 20 strains of an as yet incompletely characterized rickettsia from O. concanensis in Oklahoma. The last strains are similar to an earlier isolate from A. cooleyi in South Dakota and may belong to the newly proposed R. belli serogroup (see Project 00065-08). Both species of argasid tick inhabit the same biocenose, i.e., cliffs and rocky outcroppings, although the vertebrate hosts are different. Isolation and characteristics of these organisms, along with the description of a R. canada-like agent isolated from I. angustus collected in the Black Hills will be reported shortly. (Yunker, Clifford, Thomas)

In the project on vector-pathogen relationships, much of the year was devoted to preparing, storing and distributing stocks of the 6 lines of cells from Dermacentor ticks (see last year's report). In order to preclude their loss, some of the lines have been sent to 12 interested investigators in the United States and abroad. HHS has filed application for a patent on these lines as substrates for pathogen growth. This has necessitated considerable preparatory effort to meet the requirements of the U.S. Patent Office and the American Type Culture Collection. This year the spectrum of susceptibility of 2 lines, D. variabilis (RML-15) and D. parumapertus (RML-16), for growth of various rickettsiae was determined. All of 4 strains of rickettsiae examined replicated in these cells, several to high levels. The same methods used to establish the Dermacentor cell lines were successful in establishing

2 lines of serially propagating cells from *Rhipicephalus sanguineus*, a cosmopolitan species of medical and veterinary importance. As evidenced by our studies of Cascade virus, use of poikilothermic cell lines, particularly of arthropod origin, are more useful than homeothermic systems in some instances for isolation and characterization of arthropod-borne agents. (Yunker)

PATHOGENESIS OF SLOW VIRAL DISEASES

All new work in this project was discontinued 3 years ago. However, several experiments with Creutzfeldt-Jakob disease (CJD) and scrapie are still in progress. To date, tissues from 4 of 10 specimens of brain from CJD patients have induced encephalopathy in goats inoculated intracerebrally 4 to 5 1/2 years earlier. The caprine disease is clinically and neurohistologically indistinguishable from caprine scrapie. The incubation periods in animals thus far affected have ranged from 37 to 56 months. At least 3 additional goats appear to be in prodromal stages of disease. If full-blown encephalopathy supervenes, 6 of the 10 brains will have induced illness. The groups that received tissues from the other 4 brains 47 months (3) and 66 months (1) ago will continue to be watched. These results suggest that scrapie virus and the putative CJD virus are closely related pathogenically as suggested by others. However, epidemiologic findings have not yet demonstrated any link between CJD and exposure to scrapie virus.

Scrapie virus directly from sheep or goat brain causes encephalopathy in mink inoculated intracerebrally after an incubation period of 12 to 17 months. This year, 4 of 6 sapphire mink that received 0.5 ml of $10^{-1.5}$ suspension of infected Targhee sheep brain developed an encephalopathy after an incubation period of 35 to 42 months. Mink similarly inoculated with brain from a Rambouillet and from a Suffolk sheep have thus far remained unaffected. This observation is of keen interest because of the long incubation period and particularly because experimental transmission of scrapie by the intranasal route has not been reported before in any animal. Epidemiologic implications under natural circumstances are uncertain, but aerosol exposure of man to this virus and related ones should be of some concern to those working with these agents in the laboratory.

Processing, examining, and evaluating the backlog of histologic material collected during the course of these studies, but peripheral to original objective of the project, continue. Most of this work concerns the general pathology of mink, particularly neoplasia. (Hadlow)

IMMUNOBIOLOGY OF RELAPSING FEVER

In studies to elucidate the pathogenesis of relapsing fever and the dynamics of seroconversion of *Borrelia hermsi*, 24 immunologically distinct serotypes were identified last year, reagents were prepared to detect serologic changes by immunofluorescence, and investigations were initiated to determine whether changes in surface antigens occur independently of host immune responses or whether seroconversion is initiated by immune reaction of the host. Evidence to date suggests that the latter hypothesis is correct, probably as a result of production of immune globulins. Initial attempts to demonstrate seroconversion by challenging the susceptibility of animals infected with a single serotype 5 days earlier and rendered free of organisms by antibiotic treatment were inconclusive. Large challenge doses were necessary before seroconversion occurred and it was difficult to prove that the challenge dose was not contaminated with a few organisms of the emergent serotype. Attempts to induce seroconversion in normal animals with mixtures of few organisms and peritoneal exudate cells from animals immune to the same serotype resulted in incomplete conversion. A few organisms of the challenge serotype were always present. Complete seroconversion was achieved by passive transfer of varying dilutions of antiserum (slightly higher than the neutralizing endpoint) followed in 4 to 5 hrs by homologous challenge with 1,000 *Borrelia* of a single serotype. Future studies will attempt to define the serum fractions responsible for conversion and to characterize the surface antigens that distinguish the 24 serotypes. (Stoenner)

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ADMINISTRATIVE REPORT

Currently, the Epidemiology Branch (EB) comprises 3 sections: Arthropod-Borne Diseases, Epidemiology, and Histopathology. The EB is manned by 7 Ph.D.-level scientists, all tenured, including 4 in the Arthropod-Borne Diseases Section, 2 in Epidemiology, and one in Histopathology. In addition, there are 7 supporting staff and one secretary assigned to the Office of the Chief. A permanent Chief of EB has not been appointed.

During the year one clerk (Val Moore), on temporary appointment, was added to the staff to curate the work on tick taxonomy. Five persons left the staff because of transfer or retirement. These persons have not been replaced. Nurse Officer, Elizabeth Casper, reached mandatory retirement on July 31, and Edward Patzer retired on disability on June 26. Both were with the Epidemiology Section. Jack Cory retired from the Arthropod-Borne Diseases Section in January and Stanley F. Hayes and Dan Corwin (Arthropod-Borne Diseases Section) were transferred to the Operations Branch on July 1 to become supporting staff for the newly established Electron Microscopy Section.

Dr. Beverly R. Norment completed his IPA assignment on June 1 and returned to Mississippi State University. Dr. William Todd's IPA assignment was extended for 1 year. However, he has also been reassigned to the newly created EM Section to work with Dr. Garon.

The EB continues to provide consultative expertise and rickettsial reference reagents to qualified foreign and domestic investigators in keeping with its role as WHO Collaborating Centre for Rickettsial Reference and Research.

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HONORS AND AWARDS

The following activities reflect recognition of EB by their peers in the scientific community.

Editorial Boards of Journals:

Dr. W. Burgdorfer - Acta Tropica
Dr. W. J. Hadlow - Fundamental and Applied Toxicology
Dr. C. E. Yunker - Journal of Parasitology

Drs. Burgdorfer, Clifford, Hadlow, Keirans, and Yunker reviewed manuscripts for J. Med. Entomol., J. Econ. Entomol., J. Parasitol., Ann. Entomol. Soc. Amer., Entomol. Soc. Wash., Ent. Soc. Amer., Can. J. Zool., Vet. Pathol., J. Infect. Dis., Infect. Immun., and Am. J. Trop. Med. Hyg.

Professional Posts:

Dr. R. N. Philip - Elected Vice-President of newly established Society for Rickettsiology and Rickettsial Diseases.

Dr. W. Burgdorfer - Director, WHO Collaborating Centre for Rickettsial Reference and Research, RML; Member of Research and Thesis Committee of the Zoological Institute, University of Neuchatel, Switzerland; Co-Chairman and Co-Editor, RML Conference on Rickettsiae and Rickettsial Diseases, Sept. 1980.

Dr. W. J. Hadlow - Adjunct Professor of Veterinary Pathology, Wash. St. Univ.

Dr. J. E. Keirans - Received grant for research at British Museum (Nat. Hist.) from Burroughs-Wellcome Co.

Invited Lectures and Participation in Meetings and Symposia

Dr. W. Burgdorfer - invited to give lecture on "The dog - a reservoir o Rocky Mountain spotted fever?" at 118th Annual Meeting of the American Veterinary Medical Association, Saint Louis, MO, July 20-23, 1981; invited to attend Rockefeller Foundation Conference on "Current Topics in Pathogen-Vector-Host Research" and present lecture on "Overview of Vectors of Animal Pathogens: Pathogen-Vector Interactions and Transmission Mechanisms"; offered a research stipend by Veterinary Research Institute, Onderstepoort, to work at Onderstepoort Laboratory in collaboration with Dr. J. D. Bezuidenhout and to establish a rickettsial research laboratory.

Honors and Awards (cont'd)

Dr. W. J. Hadlow - invited to present lecture on "Pathogenesis of Scrapie", School of Public Health, University of California, Berkeley, and Veterans Administration Medical Center, San Francisco; invited to present lecture on "Slow Viral Diseases", Davis Foundation for Advancement of Veterinary Pathology, Fifth Annual Symposium, Bozeman, MT.

Dr. C. E. Yunker - Invited to present a seminar on preparation and application of tick tissue cultures at Old Dominion Univ., Norfolk, Va.; invited to prepare a chapter on diseases of laboratory animals for a CRC Handbook on Arachnids and Disease"; invited to participate in a symposium on "Culture of cells from invertebrates other than insects" at Meetings of American Tissue Culture Association, Washington, D.C., June 8, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00061-19 EB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Natural History of Tick-Borne Rickettsial Agents and Their Public Health Significance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID
OTHER:	R. N. Philip	Medical Director	EB NIAID
	E. A. Casper	Nurse Director	EB NIAID
	J. C. Williams	Biochemist	LMSF NIAID

COOPERATING UNITS (if any) Dr. J. N. MacCormack, CDC Branch, Dept. Human Resources, North Carolina; Drs. J. Anderson and L. Magnarelli, Conn. Agric. Res. Exp. Station; Dr. A. Aeschlimann, University of Neuchâtel, Switzerland.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.1	1.2	2.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with studies of Rocky Mountain spotted fever and other tick-borne rickettsial diseases in the United States and in other countries regarding ecology, identification, and characterization of the rickettsial agent(s) with their associated vectors. With collaboration of outside agencies (state health departments, public health laboratories, hospitals, physicians, etc.) source material for comparative studies on the various rickettsial types and information about the distribution of vector species associated with spotted fever in the United States is obtained. This project also considers the cellular and subcellular aspects of interaction between tick-borne agents, particularly rickettsiae, and their vectors.

PHS-6040
(Rev. 10-76)

Project Description:

Rocky Mountain spotted fever in the U.S. reached a record high (1,156 cases) during the past year and so far in 1981 exceeds by 26% the number of cases reported as of the same date last year. Many requests for assistance and cooperative ecological research had to be denied because of our continued shift toward molecular biology of rickettsial agents and their relationship(s) to tick vectors. Research priority was assigned to two major projects: 1) evaluation of dogs as sources for infecting ticks with Rickettsia rickettsii and other spotted fever-group agents, and 2) the "interference" phenomenon as a possible means to limit and/or prevent distribution of infected ticks. Cooperative studies with the North Carolina Department of Human Resources, the Connecticut Agricultural Experiment Station, and the University of Neuchâtel, Switzerland, were continued on a very limited scale and will be terminated as soon as research findings are completed for publication.

Research accomplishments during the past year include the following findings:

The role of dogs as sources for infecting ticks with spotted fever-group rickettsiae (R. rickettsii, R. rhipicephali, R. montana) was evaluated quantitatively following infection by syringe or by tick bite. Dogs inoculated with 1,000 to 100,000 Vero cell-culture PFU of virulent R. rickettsii (Sawtooth ♀ 2, Wachsmuth) or fed upon by ticks infected with this agent responded with rickettsemias lasting from 4 to 7 days with rickettsial concentrations sufficient to infect a small percentage (3 of 348 or 0.8%) of simultaneously feeding normal ticks. Dogs infected with Sawtooth ♀ 2 strain became ill with short-term fever, anorexia, lethargy, and conjunctivitis whereas dogs infected with the spotted fever serotypes, R. rhipicephali and R. montana, neither developed detectable ricettsemias nor clinical signs of illness. Contrary to reports in recent literature, dogs do not serve as efficient sources for infecting ticks with spotted fever rickettsiae in nature.

In southern Connecticut, dogs were found to serve as suitable enzootic or epizootic indicators of rickettsial activity. Of 1,576 dog sera screened by microimmunofluorescence, 174 (11%) contained specific antibodies at titers $\geq$1:64 against R. rickettsii (n = 31), R. montana (n = 34), R. rhipicephali (n = 19) or the unclassified 369C rickettsia (n = 90).

Columbian ground squirrels (Spermophilis col. columbianus), golden-mantled ground squirrels (S. lateralis tescorum) and chipmunks (Eutamias spp.), all efficient sources for the infection with R. rickettsii of the wood tick, Dermacentor andersoni in western Montana, proved to be nonsusceptible to R. rhipicephali and R. montana, two serotypes commonly associated with D. andersoni in that region. This suggests that murine rodents (mice, voles, rats) are the main reservoirs of these serotypes. Although R. montana was originally isolated from the tissues of voles (*Microtus pennsylvanicus*), the host association of *R. rhipicephali* is still unknown.

Additional experiments were carried out to further substantiate "interference" between spotted fever-group rickettsiae (See Annual Report 1980). Again, it was not possible to super-infect with virulent *R. rickettsii*, larval ticks infected ovarially with either *R. montana* or *R. rhipicephali*. Similarly, *D. marginatus* infected with *R. slovaca* could not be super-infected with *R. rickettsii*. Normal *D. marginatus*, on the other hand, showed *R. rickettsii* infection rates in excess of 90%.

Interference appears to be related to cellular effects preventing the secondarily acquired rickettsiae from adsorption to the surface of already infected cells. Studies are in progress with persistently infected vole tissue cultures to analyze ultrastructurally the mechanism(s) of this phenomenon which appears to be an important factor responsible for the focal nature or even absence of *R. rickettsii* in areas where *R. montana*, *R. rhipicephali* or other as yet unidentified nonpathogenic spotted fever-group serotypes are abundant.

It is of interest that the 369C agent, a rickettsialike microorganism in many ticks (*D. andersoni*, *D. variabilis*) throughout the U.S., does not interfere with *R. rickettsii*. Recent biochemical analyses of this agent (see Project No. Z01 AI 00065-08) indicated that this organism is not a member of the spotted fever biogroup.

Theoretically, interference provides a biological control measure for eventual elimination of *R. rickettsii*-infected ticks.

Results of continued investigations, including electron microscopy concerning "sexual transmission" of *R. rickettsii*, indicate that this phenomenon is not a viable mechanism for the maintenance and distribution of spotted fever rickettsiae in nature. Even though rickettsiae are detectable in early and primary spermatocytes of infected *D. andersoni* males, there is no evidence of rickettsial survival during development of spermatozoa. Vacuolized and degenerating rickettsiae are occasionally found in late spermatids but not beyond this stage of development. Examination of the progeny of tick females mated by infected males have been uniformly negative. Thus, it appears that "sexual transmission" of *R. rickettsii* in *D. andersoni* consists, as pointed out previously, of a mechanical transfer of rickettsiae in epithelial-type cells and in the fluids of the spermatophores of infected males. It does not lead to a generalized infection of the female and to the progeny.

Ultrastructural analysis by high voltage electron microscopy of persistently infected vole cell cultures led to the detection of extensions of the rickettsial outer-cell membrane which appear to interact with the cytoskeletal network of host cells. The nature of these fiberlike structures is under investigation.

Ultrastructural findings related to wolbachiae in Rhipicephalus sanguineus suggest existence of a chlamydialike developmental cycle for these symbiotes. Elementary bodylike structures have been identified and are currently being investigated.

The second phase of the cooperative study with the University of Neuchâtel, Switzerland, on the ecology of tickborne rickettsiae in that country evaluated a variety of rodent species as possible reservoirs of the "Swiss agent" and of R. slovaca for infecting the respective tick vectors, Ixodes ricinus and D. marginatus. Serologic surveys in the two major study areas (Staatswald, Val de Maggia) suggest exposure to the Swiss agent in Apodemus, Clethrionomys, and Microtus spp., and to R. slovaca in Apodemus. Experimental studies in the laboratory have so far shown the susceptibility of Apodemus and Microtus to the Swiss agent.

The PI of this project continued to serve as the Director of RML's WHO Collaborating Centre for Rickettsial Reference and Research. As every year, the Centre provided reference reagents and expertise, particularly in tickborne rickettsial diseases and Q fever, to various domestic and foreign health agencies and research institutions.

Publications:

Burgdorfer, W.: Borreliae. In Lennette, E., Balows, A., Hausler, W. T., Jr., and Truant, T. P. (Eds.): Manual of Clinical Microbiology, 3rd ed. Washington, D.C., American Society for Microbiology, 1980, pp. 383-388.

Burgdorfer, W., Cooney, J. C., Mavros, A. J., Jellison, W. L., and Maser, C.: The role of cottontail rabbits (Sylvilagus spp.) in the ecology of Rickettsia rickettsii in the United States. Am. J. Trop. Med. Hyg. 29: 686-690, 1980.

Feng, W. C., Murray, E. S., Burgdorfer, W., Spielman, J. M., Rosenberg, G., Dang, K., Smith, C., Spickert, C., and Waner, J.: Spotted fever group rickettsiae in Dermacentor variabilis from Cape Cod, Massachusetts. Am. J. Trop. Med. Hyg. 29: 691-694, 1980.

Burgdorfer, W.: The arthropod vector as a selective transport system of animal and human pathogens. In McKelvey, J. J., Jr., Eldridge, B. F., and Maramorosch, K. (Eds.): Vectors of Disease Agents. Interactions with Plants, Animals and Man. New York, Praeger Publishers, 1981, pp. 44-60.

Péter, O., Burgdorfer, W., and Aeschlimann, A.: Enquête épidémiologique dans un foyer naturel de Rickettsies à Ixodes ricinus du plateau suisse. Ann. Parasitol. Hum. Comp. 56: 1-8, 1981.

Magnarelli, L. A., Anderson, J. F., Philip, R. N., Burgdorfer, W., and Casper, E. A.: Endemicity of spotted fever-group rickettsiae in Connecticut. Am. J. Trop. Med. Hyg. 30: 715-721, 1981.

In press:

Burgdorfer, W.: Rickettsioses. In Hubbert, McCulloch and Schnurrenberger (Eds.): Supplementum to "Diseases Transmitted from Animals to Man".

Anderson, J. F., Magnarelli, L. A., Burgdorfer, W., Casper, E. A., and Philip, R. N.: Importation into the United States from Africa of *Rhipicephalus simus* on a boutonneuse fever patient. Am. J. Trop. Med. Hyg.

Graf, J.-F., Péter, O., Burgdorfer, W., and Aeschlimann, A.: Présence d'une Rickettsie du group "spotted fever" chez *Amblyomma variegatum* de Côte d'Ivoire. Ann. Univ. Abidjan

Todd, W. J. and Burgdorfer, W.: Rapid processing of biopsy specimens for examination by electron microscopy. Am. J. Clin. Pathol.

Burgdorfer, W., Hayes, S. F., Thomas, L. A., and Lancaster, J. L., Jr.: A new spotted fever group rickettsia from the Lone Star tick, *Amblyomma americanum*. In Burgdorfer, W. and Anacker, R. L. (Eds.): Rickettsiae and Rickettsial Diseases. Academic Press

Burgdorfer, W., Hayes, S. F., and Mavros, A. J.: Nonpathogenic rickettsiae in *Dermacentor andersoni*: A limiting factor for the distribution of *Rickettsia rickettsii*. Ibid.

Hayes, S. F. and Burgdorfer, W.: Ultrastructural comparisons of wolbachia-like symbiotes of ticks (Acari: Ixodidae). Ibid.

Magnarelli, L. A., Anderson, J. F., and Burgdorfer, W.: Geographic distribution of human cases of Rocky Mountain spotted fever, infected ticks, and seropositive mammals in Connecticut. Ibid.

Todd, W. J., Burgdorfer, W., Mavros, A. J., and Wray, G. P.: Ultrastructural analysis of *Rickettsia rickettsii* in cultures of persistently infected vole cells. Ibid.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00063-11 EB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Immune Responses to Rickettsial Infections and Legionnaires' Bacterium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. A. Thomas	Res. Microbiologist	EB NIAID
	M. G. Peacock	Microbiologist	EB NIAID
OTHER:	R. N. Philip	Medical Director	EB NIAID
	E. A. Casper	Nurse Director	EB NIAID
	J. C. Williams	Biochemist	LMSF NIAID

COOPERATING UNITS (if any) Dr. Gordon Meiklejohn, University of Colorado Med. Center, Denver; Dr. Donald Waldhalm, Vet. Research Lab., Caldwell, ID; Dr. Tom McCaul, London School of Hygiene and Tropical Medicine.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Epidemiology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: 0.6	OTHER: 0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study immune responses in man and experimental animals to natural and experimental rickettsial infections. We conducted a serosurvey of a mini-epidemic of Q fever in personnel at the University of Colorado Medical Center and an outbreak of abortion in dairy goats in Idaho attributed to Coxiella burnetii. This past year we also studied the morphological changes of the Legionnaires' disease bacterium during growth in liquid culture media using scanning electron microscopy and investigated the use of acridine orange as a vital stain for Legionella pneumophila. Another study used the guinea pig as an experimental model to determine serologic variation in strains of L. pneumophila, previously differentiated and assigned to serogroups by direct immunofluorescence using conjugated immune rabbit sera. Serologic reactions were measured by enzyme-linked immunosorbent assay (ELISA) using soluble antigen and the microagglutination (MA) test using particulate antigen.

PHS-6040
(Rev. 10-76)

Project Description:

Q fever. Although Coxiella burnetii is believed to be nonpathogenic for domestic livestock, investigators in the U.S. and Europe have attributed abortions in goats and sheep to infection with the organims. Other workers in this country have shown that cattle vaccinated with formalin-inactivated antigen and then challenged with a large challenge dose had normal full-term calves, whereas nonvaccinated cows aborted late in pregnancy.

Recently (1980-1981), sporadic cases and mini-epidemics of Q fever occurred in universities and private laboratories in California, Colorado and Connecticut. Q fever poses a serious health hazard to people who use sheep or neonatal lambs in research centers which process infected animals or their by-products. C. burnetii in titers to 10^8 guinea pig infectious doses per gram of sheep placental cotyledon tissues is a common observation.

In collaboration with Dr. Waldhalm, we have investigated an outbreak of abortion in dairy goats. Direct FA staining of impression smears of placental cotyledons from aborted goat fetuses revealed massive infection by C. burnetii. Fifty-four of 58 mature does aborted fetuses in this study. Only 24 of 119 animals in this dairy flock did not show significant antibodies to C. burnetii. A serosurvey of microagglutination (MA), complement-fixation (CF) and microimmunofluorescence (micro-IF) tests revealed all the other animals in this herd had high titers to both phase I and phase II C. burnetii. C. burnetii was isolated from an aborted fetus from this dairy goat herd. In view of the growing popularity of backyard goat flocks as a milk supply for their owners, more attention should be given to controlling C. burnetii infections in goats.

In a collaborative study with Dr. Meiklejohn at the University of Colorado Medical Center, we have completed a serosurvey of Q fever antibodies. Sixty-five of 107 university employees paired sera had significant titers for C. burnetii by either the MA, C, and micro-IF tests. Dr. Meiklejohn is now correlating clinical disease and seroconversion. All soil and dust samples from the research area were negative for C. burnetii seroconversion in intraperitoneally inoculated guinea pigs.

Serum samples taken in August at this research institution from a representative number of pregnant ewes and goats indicated significant antibody titers in 67% of the animals tested. It was presumed that ovine surgery and utilization of pregnant ewes in ongoing research projects at the University of Colorado were responsible for the Q fever epidemic.

Legionnaires' disease. The Philadelphia 2 strain of Legionella pneumophila was grown aerobically in liquid yeast extract broth media with varying concentrations of glucose and glutamate. Under conditions of mild aeration, 1% glucose concentration caused spectacular early growth in the liquid media compared to media without its inclusion. However, under conditions of vigorous aeration, no significant differences were noted in growth, whether glucose or glutamate were incorporated.

Morphological changes of the Legionnaires' disease bacterium (LDB) at 16, 24, 30, 40, 51 and 93 hours were observed with scanning electron microscopy. The organism displayed a smooth appearance at 16 hours of culture with considerable pleomorphism at 24 hours which progressed to a wrinkled surface and surface blebing at 51 hours to degenerated forms in all later subculture. At least three cellular antigens were released into the growth media. One of the antigens was the major cell surface antigen.

Acridine orange was found to be a useful vital stain and may be useful in observing the organism in tissues. There was considerable discrepancy between colony forming units and the total number of bacteria as determined by turbidity or by direct bacterial counting. Only rare organisms in late logarithmic stages stained with acridine orange fluoresced a brilliant orange with epifluorescent microscopy, whereas all organisms were brilliant-orange in early subculture. Correlation of colony forming ability and brilliant-orange fluorescence was substantiated. The use of acridine orange staining may be useful as a vital stain for rickettsial research. (Peacock)

Both soluble and particulate antigens were prepared from cultures of 10 strains of L. pneumophila, representing 6 serogroups, grown on charcoal yeast extract agar. Guinea pigs (6 per group) were inoculated intraperitoneally with 50 μg of inactivated or viable organisms in each of 6 or 7 doses over a period of 80 days. The animals were bled at intervals from 10 to 450 days. Uninoculated guinea pigs were maintained as sentinel animals to determine possible cross infection. Sera from all guinea pigs were tested individually by the MA test. Subsequently, sera were pooled according to strain and bleeding date and the serum pools were tested by MA and ELISA.

Significant findings are summarized as follows: 1) Homologous antibody of significant titer (both MA and ELISA) was detected against each strain at the first bleeding (10 days). 2) Significant variation in reaction patterns (both MA and ELISA) was obtained in guinea pigs inoculated with different strains of serogroup I (Knoxville, LAC, LAP, LAW) and also with strains of serogroup IV (LA, LAB). In some instances, it was difficult to assign a strain to a particular serogroup. 3) Higher titers were obtained in most instances with ELISA than with MA. 4) Serogroup I antisera showed little or no reactivity with serogroups II, III, IV and V. However, low ELISA titers were obtained against serogroup VI antigen (Chicago-2). 5) Serogroup II (Togus strain) antisera were nonreactive to serogroups I and V, but are broadly reactive to serogroups III, IV, and VI. 6) Serogroup III (Bloomington strain) was significantly reactive, either by MA or by ELISA to all serogroups except V (Dallas-IE strain). Cross-reactivity was detected in some instances by MA and not by ELISA, while in other cases the reverse was noted. 7) Serogroup IV (strains LA and LAB) showed little or no cross-reactivity with strains of serogroups I and V; however, there were significant cross-reactions with serogroups II, III, and VI by ELISA. Little or no cross-reactions were noted by MA. 8) None of the sera of serogroups I, II, III, or IV reacted significantly with antigen of serogroup V. 9) Sera of all 4 serogroups were moderately reactive by ELISA against antigen of serogroup VI; however, there was little or no cross-reactivity by MA. 10) Homologous antibody persisted in moderate titer for as long as 1 year after last inoculation. 11) Sentinel animals did not develop antibody against any of the 8 strains being worked with.

This investigation revealed that it is important to use more than one antigen (possibly a polyvalent antigen representing different serogroups) when testing guinea pig sera and perhaps human sera for antibody against Legionnaires' disease. It also showed that more than one serological test should be used when testing such sera since cross-reactions may not be detected by one test only. The time following initial exposure or onset of illness is a factor to be considered in determining the serological method(s) to be employed in antibody measurement. (Thomas)

Results of the completed studies are being readied for publication. Basic research on Legionnaires' disease has been terminated in this laboratory.

Publications:

Ormsbee, R. A., Peacock, M. G., Bickel, W. D., and Fiset, P.: A comparison of some biologic characteristics of isolates of the Legionnaires' disease bacterium. Ann. Clin. Lab. Sci. 11: 53-62, 1981.

In press:

Kimbrough, R. E., III, Ormsbee, R. A., and Peacock, M. G.: Q fever endocarditis: a three and one-half year follow-up. In Burgdorfer, W. and Anacker, R. L. (Eds.): Rickettsiae and Rickettsial Diseases. Academic Press

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00065-08 EB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Antigens and Classification of the Rickettsiae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. N. Philip	Medical Director	EB NIAID
	R. L. Anacker	Research Microbiologist	LMSF NIAID
OTHER:	E. A. Casper	Nurse Director	EB NIAID
	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID
	C. E. Yunker	Scientist Director	EB NIAID

(see same project number in LMSF report)

COOPERATING UNITS (if any) Dr. R. S. Lane, University of California, Berkeley; Dr. S. W. Gordon, Ohio Dept. of Health, Columbus.

LAB/BRANCH

Epidemiology Branch & Laboratory of Microbial Structure & Function (RML)
Hamilton, MT 59840

SECTION

Epidemiology Section and Rickettsial Diseases Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-range objectives of this project are to develop and apply practicable procedures for classification of spotted fever- and typhus-group rickettsiae and to determine the nature and biological properties of rickettsial antigens and constituents, particularly those that are associated with pathogenicity. This part of the project focuses on differentiating serologically the kinds of rickettsiae found in arthropod vectors known to transmit rickettsial disease agents to man or are involved in the maintenance of pathogenic rickettsiae in nature with particular reference to the United States. The hemolymph test (HT) for preliminary screening of ticks, Vero-cell culture for isolation of rickettsiae from HT-positive ticks, and the microimmunofluorescence (micro-IF) test of mouse antisera for identification of isolates have been excellent procedures for this purpose. Representative strains of the important serotypes are being examined by other techniques for biologic characterization (see also same project number in LMSF report).

Project Description:

1. California studies. The 1980 studies described in last year's report were completed and reported at the 1980 Rickettsiology Conference in October. Of 1,065 ticks representing one argasid and 6 ixodid species from 11 counties, 181 (all ixodid spp.) from 7 counties were HT-positive. Sixty-four isolates of rickettsiae were recovered in Vero-cell cultures from Dermacentor occidentalis, D. parumapertus, D. variabilis, Haemaphysalis leporispalustris, and Ixodes pacificus. These comprised 5 serotypes, 3 of which belonged to the spotted fever group (Rickettsia rhipicephali, unclassified 364D-like strains, and unclassified Tillamook-like isolates), and 2 of which the biotype is uncertain.

One of the latter, an R. canada-like strain (CA410), was confirmed in further studies as being indistinguishable from the R. canada prototype #2678 recovered in Ontario, Canada, in 1963. Phenotypic properties included pathogenicity in cell culture, embryonated eggs, guinea pigs, and microtus, serologic crossreactions in several species, streptomycin resistance, and EM ultrastructure. In addition, the DNA G + C base ratios (about 30%) were indistinguishable (see same project number in LMSF report). The significance of finding a second strain is that R. canada is a stable species, it spans broad geographic areas and may be present in the natural arthropod host, H. leporispalustris, throughout its range from Alaska to Argentina. The recent findings of Myers et al. that R. canada has similar DNA base composition to R. prowazekii and R. typhi, but a significantly different genome size and low DNA:DNA homology indicates that R. canada probably belongs to a distinctive biogroup.

The fifth serologic group from California comprised 369C-like isolates from H. leporispalustris, D. occidentalis, D. parumapertus, and D. variabilis (see below).

2. R. belli. During the last 7 years some 225 isolates were obtained from ticks that crossreacted in tests of mouse antisera with an unclassified rickettsia designated as 369C but did not react with any other rickettsial serotype. These strains were thought to be members of the spotted fever group (SFG) on basis of their strong crossreactions with other SFG rickettsiae in tests of antisera from higher species including man. However, during this last year, strong reactivity against 369C, but little or none against spotted fever rickettsiae, was detected in sera from all patients convalescing from epidemic and murine typhus. It then became obvious that 369C shared one or more antigens with both the spotted fever and typhus groups. However, other biologic properties set it apart from other species of rickettsiae including morphology and ultrastructure, spectrum of pathogenicity for laboratory animals, antibiotic sensitivity, and behavior in cell culture. DNA base determinations showed the G + C composition to be similar to that of the typhus group (about 30) (see also same project number in LMSF report).

However, genome size and the degree of DNA hybridization with other rickettsiae have not been determined. On basis of the distinct phenotypic differences, 369C has been designated as the prototype strain of a new species of rickettsia, *R. belli*, coined after the rickettsiologist (E. J. Bell) who first isolated the organism in 1966. Isolates of the *R. belli* serogroup have been recovered from 8 species of ticks (2 families; 4 genera) including the major vectors of Rocky Mountain spotted fever, collected from 8 states. There is no evidence to implicate this organism as a source of human disease, but because of its frequency and shared antigen(s) with the spotted fever group, it can be mistaken for *R. rickettsii* in HT of ticks taken from patients with Rocky Mountain spotted fever.

3. *Ohio tick survey*. In collaboration with Dr. Gordon, we attempted to isolate rickettsiae from HT-positive ticks collected in 11 counties, 1978-80. Eighty-one strains of rickettsiae were recovered and identified. Seventy-nine were typed as *R. belli*, 2 as *R. montana* and one as *R. rickettsii*. Many of the ticks were collected from what had been an RMSF focus several years earlier in suburban Columbus. It was surprising that more isolates of *R. rickettsii* were not obtained.

We now have a reasonably complete knowledge of the kinds of rickettsiae that systemically infect three major vectors of RMSF in the United States including *D. andersoni*, *D. variabilis*, and *D. occidentalis* and have established a solid base for systematic sorting and characterization of biochemical, genetic, and ecologic interrelationships among these organisms. Except for identification of strains isolated from ticks collected this year in North Carolina, the survey-type approach will be curtailed. Our 7-year experience will be readied for publication.

Publications:

Philip, R. N. and Casper, E. A.: Serotypes of spotted fever group rickettsiae isolated from *Dermacentor andersoni* (Stiles) ticks in western Montana. *Am. J. Trop. Med. Hyg*. 30: 230-238, 1981.

Philip, R. N., Lane, R. S., and Casper, E. A.: Serotypes of tick-borne spotted fever group rickettsiae from western California. *Am. J. Trop. Med. Hyg*. 30: 722-727, 1981.

In press:

Lane, R. S., Philip, R. N., and Casper, E. A.: Ecology of tick-borne agents in California. II. Further observations on rickettsiae. In Burgdorfer, W. and Anacker, R. L. (Eds.): *Rickettsiae and Rickettsial Diseases*. Academic Press

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00069-20 EB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Systematics and Vector Relationships of Certain Parasitic Arthropods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. M. Clifford	Scientist Director	EB NIAID
OTHER:	J. E. Keirans	Res. Entomologist (Med.)	EB NIAID
	C. E. Yunker	Scientist Director	EB NIAID

COOPERATING UNITS (if any) Drs. H. Hoogstraal, NAMRU-3; D. Sonenshine and P. Homsher, Old Dominion Univ.; P. Teel, Texas A&M; F. S. Lukoschus, Catholic Univ., Netherlands; Miss Jane Walker, Div. of Vet. Services, Onderstepoort; Mr. Rupert Pegram, Tick Diseases Unit, Lusaka, Zambia.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 4.2	PROFESSIONAL: 2.0	OTHER: 2.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The activities of this project currently comprise four main functions: 1) Identification of ticks received from various individuals and government agencies throughout the world. Only one other institution in the world is capable of performing this service. 2) Systematic study of certain groups of parasitic arthropods. The foremost tool in systematic studies, the scanning electron microscope, has greatly aided in elucidating taxonomic concepts in acarines actually or potentially involved in transmission of disease agents. 3) Retrieval and use of tick data in the Smithsonian data retrieval system. Installation of a mini-computer-word processor system at RML to communicate with NIAID and Smithsonian computers. 4) Colonization of medically important arthropod vectors, which are furnished to outside investigators.

PHS-6040
(Rev. 10-76)

Project Description:

The primary object of this project is the systematic treatment, including taxonomy and classification, biology, ecology and colonization of parasitic arthropods of known or potential medical importance. The Rocky Mountain Laboratories' tick collection contains 20 tick genera and is the only collection in the world that has all of the described genera. In species it is represented by roughly 88% of the currently recognized species from all zoogeographical areas of the world and deposited here are 245 types (holotypes, paratypes, lectotypes, etc.). Recognizing that a well-documented specimen collection is necessary to the proper practice of medical and veterinary parasitology, the American Society of Parasitologists has designated the Rocky Mountain Laboratories' tick collection a national resource center for type and voucher specimens, specimen data and related literature.

Argasid ticks: Publication of our manuscripts on Haller's organs of the genus _Argas_ has been delayed because more species have been examined. We hope to complete this project in the coming year and proceed to the genus _Ornithodoros_.

Some work was also accomplished on our ongoing studies of the _Ornithodoros capensis_ complex. _O. muesebecki_ has been redescribed and SEM photos to illustrate this species are completed. We hope to work on the species related to _O. denmarki_ in the coming year.

Ixodid ticks: A study on the _Ixodes_ (_Afrixodes_) _oldi_ complex of ticks from sub-Saharan Africa has resulted in the recognition of five new species. We have characterized the three known species, described the five new species, and a manuscript including 80 scanning electron photomicrographs has been completed.

Our studies on the medically and agriculturally important genus _Rhipicephalus_ have continued. During this year an extensive review of the _R. simus_ complex was undertaken. It now appears that there are at least three and possibly four species in this complex. Scanning electron photographs have been prepared and material from many different localities is being studied.

During the year a large and important collection of ticks from Iraq was identified. This collection was important because so few ticks are seen from this region due to political unrest. Several species new to the RML collection were obtained from this study.

Extensive collections of ticks from Mali were identified during the year. This work was in collaboration with Drs. Matthysse and Teel of Texas A&M University. Vast numbers of ticks have been received since the first identifications were made and these investigators have requested that someone from the RML travel to Texas A&M to assist them.

A large part of the time of the professional staff in this unit is spent collaborating with scientists around the world. Thousands of ticks are identified and these efforts have resulted in numerous joint publications.

RML-Smithsonian computer data. In May 1981, a Datapoint 1800 Dispersed Processor with diskette and printer were installed which now enables us to communicate with the RML data base at the National Museum of Natural History and at NIH. In late July the computer center at NIH is sending out a data management expert to train us in the various uses of this equipment. We have a backlog of approximately 10,000 new tick records plus numerous corrections on our existing data base which need to be entered into the Smithsonian-NIH data file.

Nuttall Collection: In October 1980 Dr. Keirans was awarded a grant from the Burroughs Wellcome Fund to complete his research at the British Museum (Natural History) on the Nuttall tick collection. An 1800-page manuscript on this collection has been completed and submitted to the trustees of the British Museum (Nat. Hist.) for publication. Unfortunately, because of budgetary restrictions, they can only publish a work of this length on microfiche.

Service-oriented activities: The RML insectary in 1980-1981 continues to maintain approximately 32 species of ticks and provides support for both RML and visiting scientists, as well as researchers in other parts of the USA. This year, on 40 occasions, we provided 76,378 ticks of 11 different species. We continue to provide, as a routine service, tick identifications for USDA and other governmental agencies. By providing this service we are able to accumulate rare and valuable specimens for our research needs.

The future course of this project will emphasize the use of the scanning electron microscope to solve taxonomic problems among various genera of ticks. Special attention will be given to groups of ticks that contain species that are proven vectors of diseases of man and domestic animals. The taxonomic aspects of this project will be increased because we are forced to discontinue most of our participation in the tickborne disease project due to the transfer of Mr. Dan Corwin from our laboratory to the Electron Microscope Unit.

Publications:

Clifford, C. M. and Hoogstraal, H.: A new species of Ixodes parasitizing the Rice Rat in the Galapagos (Ixodoidea: Ixodidae). Proc. Entomol. Soc. Wash. 82: 378-383, 1980.

Keirans, J. E., Clifford, C. M., and Hoogstraal, H.: Identity of the nymphs and adults of the Galapagos iguanid lizard parasites, Ornithodoros (Alectorobius) darwini and O. (A.) galapagensis (Ixodoidea: Argasidae). J. Med. Entomol. 17: 427-438, 1980.

Yunker, C. E. and Radovsky, F. J.: Parasitic mites of Surinam. XXXVI. A new genus and two new species of Neotropical Macronyssidae (Acari: Mesostigmata). J. Med. Entomol. 17: 545-554, 2980.

Anderson, J. F., Magnarelli, L. A., and Keirans, J. E.: *Aponomma quadricavum* (Acari: Ixodidae) collected from an imported boa, *Epicrates striatus*, in Connecticut. J. Med. Entomol. 18: 123-125, 1981.

Hoogstraal, H., Clifford, C. M., Keirans, J. E., and Wassef, H. Y.: Recent developments in biomedical knowledge of *Argas* ticks (Ixodoidea: Argasidae). In Rodriguez, J. G. (Ed.): Recent Advances in Acarology, Vol. II. New York, Academic Press, 1979, pp. 269-278.

Homsher, P. J. and Yunker, C. E.: Bilateral gynandromorphism in *Dermacentor andersoni* (Acarina: Ixodidae): morphologic and cytogenic analysis. J. Med. Entomol. 18: 89-91, 1981.

Yunker, C. E., Binninger, C. E., Keirans, J. E., Beecham, J., and Schlegel, M.: Clinical mange of the black bear, *Ursus americanus*, associated with *Ursicoptes americanus* (Acari: Audycoptidae). J. Wildl. Dis. 16: 347-356, 1980.

Fain, A. and Yunker, C. E.: *Lutracarus canadensis*, n. g., n. sp. (Acari, Listrophoridae) from the river otter, *Lutra canadensis*. J. Med. Entomol. 17: 424-426, 1980.

In press:

Keirans, J. E.: The tick collection (Acarina: Ixodoidea) of the Hon. Nathaniel Charles Rothschild deposited in the Nuttall and general collections of the British Museum (Natural History). Bull. Brit. Mus. Nat. Hist. (Zool.)

Stark, H. E., Inlao, I., Clifford, C. M., Keirans, J. E., and Lakshana, P.: A preliminary checklist of the ticks of Thailand. Appl. Sci. Res. Corp J. Thailand

Keirans, J. E., Clifford, C. M., and Yunker, C. E.: Rocky Mountain Laboratory reference collection of ticks and parasitic mites (Acari). A Guide to the Parasite Collections of the World

Keirans, J. E.: George Henry Falkner Nuttall and the Nuttall Tick Catalogue. Bull. British Museum Nat. Hist. (Zool.)

Keirans, J. E. and Brewster, B. E.: The Nuttall and British Museum (Natural History) tick collections: Lectotype designations for ticks (Acarina: Ixodoidea) described by Nuttall, Warburton, Cooper and Robinson. Bull. British Museum Nat. Hist. (Zool.)

Spickett, A. M., Keirans, J. E., Norval, R.A.I., and Clifford, C. M.: Ixodes (Afrixodes) matopi, n. sp. (Acarina: Ixodoidea): A tick found aggregating on anti-orbital gland scent marks of the Klipspringer in Zimbabwe. Onderstepoort J. Vet. Res.

Keirans, J. E.: Rocky Mountain wood tick and American dog tick. Great Plains Beef-Cow-Calf Handbook.

Appendix 1. PL-480 Project 03-063N. Epidemiological and Biological Aspects of Tick-Borne Ifections in Egypt and Elsewhere, NAMRU-3, Cairo, Egypt

Annual Funding: $123,000

In March 1981 Dr. Keirans made a final Project Officer visit to NAMRU-3, Agouza Virus Laboratory, and Cairo University. The research portions of this project conducted at Agouza Virus Lab and Cairo University terminated as of May 31, 1981. The bibliography portion of the work conducted at NAMRU-3 is continuing at a reduced funding level.

NAMRU-3: The Bibliography Section during the past year received, reviewed, and catalogued about 800 reprints on ticks and tickborne diseases. Volume 6 of the author index was completed and sent to the publisher in March 1981. Sixty translations, mostly from Russian, were translated and a 220-page book on Omsk Hemorrhagic Fever was translated.

Agouza Virus Lab: A seroepidemiological survey was conducted on 361 rodent sera from Alexandria, Suez, and Cairo Governorates for 11 tickborne viruses previously reported from Egypt. There was a high (19.9%) prevalence rate of CF antibodies against Matariya virus, most from R. norvegicus. There was a 12.5% rate of antibodies against Wad Medani virus and overall rates between 7.7% and 4.4% were recorded for Dhori, Wanowrie, Thogoto, Dugbe, Crimean-Congo hemorrhagic fever, and Quaranfil viruses.

Cairo University: The studies undertaken at theis university focused primarily upon haematological aspects of domestic hosts for ticks. Sets of blood smears were prepared from different animals, cattle buffaloes, sheep, goats, horses, dogs, rabbits, pigs, chicken, laboratory hamsters, guinea pigs, rats, and mice. In addition, for the past year they have been studying exudates from fed and unfed ticks. Haemocytes of uninfected Argas persicus and Hyalomma dromedarii in different developmental stages and sexes were studied to determine quantitative and qualitative properties of these cells.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER
		Z01 AI 00080-14 EB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Tickborne Disease Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. E. Yunker	Scientist Director	EB NIAID
OTHER:	C. M. Clifford	Scientist Director	EB NIAID
	J. E. Keirans	Res. Entomologist (Med.)	EB NIAID
	R. N. Philip	Medical Director	EB NIAID
	L. A. Thomas	Research Microbiologist	EB NIAID

COOPERATING UNITS (if any) Dr. C. Chastel, Faculte de Medecine, Brest; Dr. H. Hoogstraal, NAMRU-3, Cairo; Dr. R. Gresbrink, Oregon State Bd. Hlth., Portland; Dr. B. Easterday, Univ. Wisc., Madison; Dr. E. Easton, S. Dakota State Univ.; Dr. J. Tully, LID, NIAID; Dr. M. Takahashi, Nat. Inst. of Health, Tokyo.

LAB/BRANCH

Epidemiology Branch (RML), Hamilton, MT 59840

SECTION

Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	0.7	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In this project the prevalence, distribution and ecology of tickborne disease agents, arboviruses, rickettsiae and spiroplasms, are studied. These pathogens are isolated in tissue cultures or laboratory animals from field-collected materials and are serologically identified. If new, pathogens are physicochemically and biologically characterized and taxonomically classified. Of particular importance is the delineation of vector-relationships and human disease potential of the increasing number of tickborne viruses in the United States.

PHS-6040
(Rev. 10-76)

Project Description:

This project is concerned with documenting the occurrence and natural history of tickborne pathogens of vertebrates, especially arboviruses. Mosquitoborne arbviruses are relatively well-studied in comparison to those that are tickborne, thus this project helps to fill an obvious void in the study of vectorborne disease. Live ticks and other materials received from collectors, including human and animal health officials in various parts of the United States and abroad, are inoculated into tissue cultures and laboratory animals. Pathogens thus isolated are identified in complement-fixation or immunofluorescence tests. Often this results in the recognition of new agents, which are then biologically, physically and chemically characterized and taxonomically classified. This year we tested over 3,200 ticks, biting insects and blood samples in 668 pools. Materials originated in 10 states of the U.S. (Alaska, California, Idaho, Montana, Oklahoma, Oregon, South Dakota, Texas, Virginia and Washington) as well as in Egypt and Paraguay. Resulting from this were 19 viral, one spiroplasmal and 21 rickettsial isolates.

Viruses. Three previously described, one new, and two as yet unidentified viruses were isolated this year. In the former group were five isolates of Yaquina Head, an Orbivirus of the Kemerovo serogroup, previously described by us from coastal Oregon, but found in the present study in seabird ticks of St. Paul I., Alaska. All Kemerovo group viruses are customarily found in migratory-bird ticks that are capable of biting man. In Central Europe and Asia, the prototype virus and related strains have caused human infections. Also recovered from the same tick species, Ixodes uriae, in bird nesting sites of St. Paul I., were three isolates of an unidentified virus of the Sakhalin group (Bunyaviridae). This agent, previously recovered by us on St. Paul I., reacts serologically with members of the serogroup from similar biotopes in the USSR and coastal Oregon, USA. However, its specific identity remains undetermined because of low reactivity of serological reagents. The presence of the above two viruses in the North Pacific points to a transoceanic continuum of tickborne viruses among migratory seabirds. The implication of this for the epidemiology of human zoonoses (e.g., influenza) warrants consideration.

Other viruses isolated this year included Quaranfil (unclassified) from Egyptian ticks, Argas arboreus, Colorado tick fever (CTF, Reoviridae) from Montana Dermacentor andersoni, Cascade (unclassified) from Oregon D. occidentalis, and an as yet unidentified agent from A. cooleyi found in gull nests in Mono Lake, California. Both Quaranfil and CTF viruses are human pathogens, and Cascade, a new virus which we reported last year, should be considered in the etiology of tick-associated human disorders of undetermined cause (see Project No. Z01 AI 00084-10 EB). A manuscript describing Cascade virus and the prevalence of this and CTF virus in man-biting ticks of Oregon is in press.

In collaboration with French and Japanese workers, we studied the identities of certain viruses of the Ornithodoros capensis complex of soft-shelled (argasid) ticks. This tick complex, which is circumglobal in the warmer latitudes, harbors its own spectrum of viruses that are ecological equivalents of the Kemerovo-Sakhalin-Uukuniemi serogroups found in hard ticks in temperate and polar regions.

One of these argasid tick viruses, Soldado, a Nairovirus of the Hughes serogroup, was recently shown by French virologists to exist as a number of antigenically distinct subtypes. We supplied these workers with our Soldado virus isolates from various geographic areas and we are now preparing specific antisera to all strains for use in serological tests. Hirota virus, recently described as a new Arenavirus by workers in Japan, was found by Yale researchers to be related to Midway virus, which had earlier been reported by us from O. capensis ticks in the Hawaiian chain. Upon request, we compared Hirota and Midway viruses in CF and indirect immunofluorescence tests and found them indistinguishable. Certain other Arenaviruses are known to cause human illnesses of epidemic proportions and high mortality.

Spiroplasms. The majority of ticks received this year were also examined for spiroplasms by inoculation into appropriate media. Last year, in collaboration with the Laboratory of Infectious Diseases, we reported the discovery of a new and serologically unique group of spiroplasms from human-biting ixodid ticks of Oregon. An additional isolation of this organism was made this year from Ixodes pacificus from Oregon. Like Cascade virus, distribution of the new spiroplasm correlates with western cases of erythema chronicum migrans and Lyme arthritis, and should be considered as a candidate causal agent.

Rickettsiae. Ixodes pacificus ticks of Oregon also yielded an isolate of the "Quarry" strain of rickettsia. This agent, a member of the spotted fever serogroup, was first reported by us from the same tick species and geographical area and has been recovered on subsequent occasions. Its repeated recovery implies a specific pathogen-vector relationship as well as a persistent enzootic focus. The 20 remaining isolates were all of an undescribed rickettsia from Ornithodoros concanensis in Oklahoma. Apparently, it represents a new rickettsial serotype, inasmuch as it is unrelated to any rickettsial agent in the RML reference collection. It was found to be indistinguishable from a unique rickettsia previously isolated by us from Argas cooleyi in South Dakota. Both A. cooleyi and O. concanensis inhabit the same biocoenose -- rocky cliffs, outcroppings and bridges where cliff-swallows and bats roost. The former tick species is a parasite of the swallow, whereas the latter feeds principally on bats. The occurrence of a rickettsia common to both arthropods may signify transfer of this pathogen between avian and mammalian hosts. A manuscript in preparation describes the isolation and characteristics of this rickettsia, the isolation of a typhus group agent near Rickettsia canada from the man-biting I. angustus, plus new vector and geographical records for other rickettsial species in American ticks.

Continuation of this project depends on the assistance, in the field, of collaborating investigators, as well as the availability of technical help. Funds for field work have not been forthcoming, and retirement or transfer of technicians without replacement have seriously affected the program.

Publications:

Tully, J. G., Rose, D. L., Yunker, C. E., and Cory, J.: Helical mycoplasmas (spiroplasmas) from *Ixodes* ticks. *Science* 212: 1043-1045, 1981.

In press:

Yunker, C. E., Cory, J., Gresbrink, R. A., Thomas, L. A., and Clifford, C. M.: Tickborne viruses in western North America. III. Viruses from man-biting ticks in Oregon. *J. Med. Entomol.*

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00081-20 EB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Epidemiology of Human Infections of Special Interest

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. N. Philip	Medical Director	EB NIAID
OTHER:	E. A. Casper	Nurse Director	EB NIAID
	L. A. Thomas	Res. Microbiologist	EB NIAID
	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID

COOPERATING UNITS (if any) Dr. R. Emmons, Viral and Rickettsial Disease Lab., Calif. Dept. of Health, Berkeley; Dr. Catherine Wilfert, Duke Univ. School of Med., Durham, NC; Dr. R. H. Schultz, Idaho Dept. of Health, Boise.

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SECTION

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NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.1	PROFESSIONAL: 1.0	OTHER: 0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project emphasizes epidemiologic investigations of infectious diseases of particular interest to the research program of this laboratory. Current investigations include studies of the etiologic relationship of various spotted fever-group serotypes to human illness, study of epidemiologic attributes pertinent to occurrence of Rocky Mountain spotted fever (RMSF) in a high incidence area, and improvement in laboratory methods for diagnosis of rickettsial infections, particularly RMSF.

1

Project Description:

1. *Etiologic role of spotted fever group (SFG) agents to human infection.* As related in last year's report antibody absorption studies were conducted on convalescent sera from 4 patients from coastal California with tickborne rickettsial disease to obtain evidence of the antigenic nature of the infecting organism and its relationship to *R. rickettsii*. One additional patient has been tested since then. Montana strains of *R. rickettsii* were clearly less effective in removing antibody from this patient's sera than were the California *R. rickettsii*-like strains. The reverse was true of a Montana patient with Rocky Mountain spotted fever (RMSF). However, this evidence is circumstantial only. Definitive identification of the etiologic agent of California spotted fever awaits isolation of rickettsiae from a patient during illness. Nevertheless it appears likely that the organisms responsible for disease in coastal counties where *Dermacentor andersoni* does not exist are the *R. rickettsii*-like strains (364D is the prototype) that are carried and transmitted by *D. occidentalis*. This organism is being given the tentative subspecies name of *Rickettsia rickettsii californica* in a manuscript being prepared for publication. Twenty-one strains of this organism have been isolated thus far from *D. occidentalis* collected in 4 different counties.

2. *RMSF, Spirit Lake, Idaho*. One of two fatal cases of RMSF serologically diagnosed by us in 1981 occurred under unusual circumstances which led to a field investigation. A 9-year-old girl living with her parents in a remote cabin, accessible only by boat, on the south shore of Spirit Lake, Kootenai County, was admitted moribund on the 10th day of illness to a Spokane, WA hospital. She died 3 days later. Paired serum samples obtained on admission and the day of death revealed an 8-fold rise in antibody titer to *R. rickettsii*. Five years earlier another fatal case of RMSF from the same county was identified by us and perusal of Idaho records for RMSF from 1911-1960 showed that 23 of the 43 cases that occurred in Kootenai Co. during this 50-year period were fatal. The case fatality rate for this county (53%) was by far the highest in Idaho (overall, 9%). Since the area where the infection was acquired could be pin-pointed to a radius of 1/2 mile from the cabin-site it was decided to conduct an intensive tick survey of the area. Although the cabin-site was situated on the north slope of heavily-forested mountainous terrain (normally poor tick habitat), we were able to collect 790 *D. andersoni* from shrubbery in clearings and on the ridge above the cabin. Twenty ticks were hemolymph test-positive, 12 females and 8 males. The females along with normal males were fed on guinea pigs, most of which died of classical RMSF. Although feeding was adequate and all females oviposited, none of the eggs was viable. Isolates were obtained from the 8 male ticks. All serotyped as *R. rickettsii* and all were extremely virulent for chick embryos and guinea pigs. Many of the HT-negative ticks were dissected but no rickettsia-like organisms were found. This is the only instance in which we have identified a focus occupied by *R. rickettsii* in absence of other rickettsiae. It is not known whether the Spirit Lake locale is a newly established site for a burgeoning tick population and invasions by *R. rickettsii* or

whether it is a relict where undefined natural barriers have precluded establishment of other SFG rickettsiae thereby fostering the maintenance of virulent R. rickettsii.

3. Other. The collaborative epidemiologic study of RMSF in two high-risk North Carolina counties has continued. Preliminary findings were presented at the Rickettsiology Conference last October. We continue to provide serologic support in testing paired sera from patients with tick-associated illnesses against a battery of rickettsial antigens. Other than the one instance mentioned last year, we have not observed antibody responses that clearly implicate rickettsiae other than R. rickettsii. It is increasingly apparent that other serotypes are seldom, if ever, etiologically involved in human disease in this area.

Publications:

Hechemy, K. E., Anacker, R. L., Philip, R. N., Kleeman, K. T., Sasowski, S. J., and Michaelson, E. E.: Detection of Rocky Mountain spotted fever antibodies by a latex agglutination test. J. Clin. Microbiol. 12: 144-150, 1980.

In press:

Davis, J. P., Wilfert, C. M., Sexton, D. J., Burgdorfer, W., Casper, E. A., and Philip, R. N.: Serologic comparison of R. rickettsii isolated from patients in North Carolina to R. rickettsii isolated from patients in Montana. In Burgdorfer, W. and Anacker, R. L. (Eds.): Rickettsiae and Rickettsial Diseases. Academic Press

Hechemy, K. E., Sasowski, S. J., Michaelson, E. E., Zdeb, M., Anacker, R. L., Philip, R. N., Kleeman, K. T., Crump, C., Kudlac, J., Joseph, J. M., Patel, J., Dowda, H., Jr., Killgore, G., Young, D., Barrick, J. H., Hindman, J. R., Elliott, L. B., and Altieri, R. H.: Rocky Mountain spotted fever: first-year evaluation of a latex agglutination test for antibodies to Rickettsia rickettsii. Ibid.

Kleeman, K. T., Hicks, J. L., Anacker, R. L., Philip, R. N., Casper, E. A., Hechemy, K. E., Wilfert, C. M., and MacCormack, J. N.: Early detection of antibody to Rickettsia rickettsii: a comparison of four serological methods, indirect hemagglutination, indirect fluorescent antibody, latex agglutination, and complement fixation. Ibid.

Wilfert, C. M., Austin, E., Dickinson, V., Kleeman, K., Hicks, J. L., MacCormack, J. N., Anacker, R., Casper, E., and Philip, R.: The incidence of Rocky Mountain spotted fever as described by prospective epidemiologic surveillance and the assessment of persistence of antibodies to R. rickettsii by indirect hemagglutination (IHA) and microimmunofluorescence (MIF) tests. Ibid.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00082-20 EB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Relation of Viruses to the Genesis of Chronic Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. J. Hadlow	Res. Veterinarian (Path.)	EB NIAID
OTHER:	None		

COOPERATING UNITS (if any) Dr. S. B. Prusiner, Dept. of Neurology, University of California, San Francisco.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Histopathology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several naturally occurring viral infections of domestic animals are excellent models for studying unusual host-virus interactions that result in slowly evolving diseases. Two of these are scrapie of sheep and goats and Aleutian disease of mink. The main concern of this project is to characterize these diseases by making observations on their clinicopathologic, virologic, and immunologic features. Such information will help define the mechanisms that give rise to protracted infectious disease not only in animals but also in man.

PHS-6040
(Rev. 10-76)

Project Description:

The intent of this project is to learn more about the unusual host-virus interactions that give rise to slowly evolving diseases after long incubation periods. Scrapie of sheep and goats and Aleutian disease of mink are studied as natural models of such diseases. Scrapie is a degenerative disease of the brain (polioencephalopathy) apparently caused by virus replicating in central nervous tissue. Aleutian disease is a chronic glomerulopathy mediated by infectious immune complexes. By the use of fairly simple methods, information is sought on the pathogenesis and natural history of each disease.

Because all new work in this project was discontinued 3 years ago by administrative decree, attention is now directed to making observations on those few experiments still in progress and to evaluating observations from earlier experiments. A few new observations were made during the past year, but little was accomplished in dealing with the raw data from the early studies.

The only active part of the original project concerns groups of goats that had been inoculated intracerebrally with brain tissue from 10 persons who died of Creutzfeldt-Jakob disease (CJD). So far, four such specimens of human brain have caused an encephalopathy in goats that is clinically and neurohistologically indistinguishable from caprine scrapie. (Three of the goats were reported last year.) The incubations periods have been 37, 43 (2), and 56 months, and the course of the disease 8 to 24 weeks. Three more goats (one from a previously positive group) now seem to be in the prodromal stage of the neurologic disease, after 48, 60, and 67 months. If full-bloom encephalopathy supervenes in them, the number of positive human brains will be 6 of 10--a result not anticipated at the outset. Goats inoculated with the other four brains will be watched for evidence of encephalopathy. One group has been inoculated 66 months, the other three, 47 months.

The significance of these observations remains as discussed last year. They suggest that scrapie virus and the putative CJD virus are closely related pathogenically, as observations by others working with nonhuman primates have also suggested. Despite this suggestion, epidemiologic findings have not yet demonstrated any link between the occurrence of CJD and exposure to scrapie virus, however indirect that may be.

As mentioned in several earlier reports, scrapie virus (in the form of sheep or goat brain suspensions) causes encephalopathy in mink inoculated intracerebrally. The incubation period by this route varies from 12 to 17 months. During the past year, 4 of 6 sapphire mink inoculated intranasally with 0.5 ml of a $10^{-1.5}$ suspension of Targhee sheep brain became affected with the encephalopathy. The disease has not appeared in mink similarly inoculated with suspensions of brain from two other sheep, a Rambouillet and a Suffolk. This observation is of special interest not only because the incubation period was unusually long (35 to 42 months), but also because experimental transmission of scrapie by the intranasal route has not been reported before in any animal. Whether this finding has any epidemiologic implications for the occurrence of scrapie in its natural hosts, the sheep and the goat, is not known. Nevertheless, aerosol exposure of man to this virus and related ones should be of some concern to persons working with these agents at the laboratory bench.

As this project continues to die and new directions are sought, adjustments must be made. Assembling and evaluating much of the old data has proved to be an onerous, time-consuming, one-man task, however conscientiously it may be approached. Too many of the early experiments were never completed, so preparing information from them for publication is not a satisfying endeavor. As a consequence, more time is being devoted to evaluating the plethora of histologic material on hand. Although much of it is peripheral to the original intent of the project, it nevertheless represents valuable material that should be dealt with as time allows. Most concerns the general pathology of mink, including in particular neoplasia. The appended list of publications attests to this direction of the project.

Publications:

Hadlow, W. J. and Ward, J. K.: Atresia of the right atrioventricular orifice in an Arabian foal. Vet. Pathol. 17: 622-637, 1980.

Hadlow, W. J.: Criteria for development of animal models of diseases of the nervous system. Am. J. Pathol. 101: S213-S219, 1980.

Hadlow, W. J., Prusiner, S. B., Kennedy, R. C., and Race, R. E.: Brain tissue from persons dying of Creutzfeldt-Jakob disease causes scrapie-like encephalopathy in goats. Ann. Neurol. 8: 628-631, 1980.

Prusiner, S., Cochran, S. P., Groth, D., Hadley, D., Martinez, H. M., and Hadlow, W.: Aging of the nervous system and prolonged incubation periods of the spongiform encephalopathies. In Amaducci, L., Davison, A. N., and Piero, A. (Eds.): Aging of the Brain and Dementia, (Aging Vol. 13). New York, Raven Press, 1980, pp. 205-216.

In press:

Hadlow, W. J.: Ocular lesions in mink affected with Aleutian disease. Vet. Pathol.

Hadlow, W. J.: Pulmonary emboli of nucleus pulposus accompanying degeneration of intervertebral disks in ranch mink. Vet. Pathol.

Hadlow, W. J. and Race, R. E.: Common occurrence of urethral cysts in older female ranch mink. Vet. Pathol.

Oliver, R. E., Gorham, J. R., Parish, S. F., Hadlow, W. J., and Narayan, O.: Ovine progressive pneumonia: pathologic and virologic studies on the naturally occurring disease. Am. J. Vet. Res.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00084-12 EB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Vector-Pathogen Relationships

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. E. Yunker	Scientist Director	EB NIAID
OTHER:	L. A. Thomas	Research Microbiologist	EB NIAID

COOPERATING UNITS (if any) Dr. Kouka Abdel-Wahab, Al Azhar University, Cairo, Egypt; Dr. Mamdouh Y. Kamel, National Research Center, Cairo, Egypt; Dr. E. Easton, S. Dakota State Univ., Brookings, SD; Dr. P. Homsher, Old Dominion Univ., Norfolk, VA.

LAB/BRANCH

Epidemiology Branch (RML), Hamilton, MT 59840

SECTION

Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.4	OTHER: 0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project deals with the interactions of vector-borne microorganisms, arboviruses, rickettsiae and protozoans, and cells of haematophagus arthropods, mainly ticks. Model in vitro systems are developed and applied to studies of pathogen host-range, growth dynamics, persistence and interference. After prolonged growth in these systems, pathogens are examined for changes in invasiveness, virulence, or antigenic characteristics. By contract with collaborative laboratories overseas, we are studying critical factors influencing survival of ticks and of pathogens within ticks from a biochemical, histochemical, and physiological standpoint.

PHS-6040
(Rev. 10-76)

Project description:

Last year we reported the establishment characterization and virus susceptibility of six lines of cells from Dermacentor ticks, a culmination of many years' efforts. This year was largely devoted to preparing, storing and distributing to interested investigators stocks of these cell lines in order to preclude their loss. Thus far, some of the lines have been sent to 12 investigators in the United States and abroad. In addition, the Office of the Secretary, HHS, has filed application for a patent on these cell lines as substrates for pathogen growth and vaccine production. This application has also entailed considerable preparatory effort in order for us to meet the requirements of the U.S. Patent Office and American Type Culture Collection, which is the official repository for patentable cell lines.

This year we tested the replication of rickettsias of various serological groups in two of the above lines, RML-15 (D. variabilis) and RML-16 (D. parumapertus). Cell cultures were inoculated with one of four rickettsiae (Rickettsia rickettsii, R. akari, R. typhi and a new agent from argasid ticks, similar to R. canada). Growth medium plus cells were assayed for rickettsial growth at weekly intervals. Growth was determined by plaque titration in Vero or Xenopus laevis (XTC-2) cells or, in the case of R. typhi, by serological conversion of intraperitoneally inoculated adult mice as measured by indirect immunofluorescence tests. All rickettsiae replicated to higher levels within one or two weeks of inoculation. For some species, this replication was as great as 1×10^5 or 1×10^6 in the relatively short time in which the tests were conducted. These results imply a potential usefulness of the tick cell lines in various aspects of research on rickettsiae and rickettsial diseases.

The same methods that we used to establish the Dermacentor cell-lines were employed to establish in serially propagating, continuous culture, two lines of cells from the brown dog tick, Rhipicephalus sanguineus, a cosmopolitan species of medical and veterinary significance.

We completed characterization of a serologically unique virus from man-biting ticks, D. occidentalis, in Oregon. Last year we reported multiple isolations of this new agent in a poikilothermic cell line (Xenopus laevis), as well as in our Dermacentor tick cell lines. This year, some physicochemical and biological parameters of the new virus, which we call Cascade, were established. The virus contains RNA and essential lipids, and is sensitive to exposure to heat and acid. Diameter of the virion is between 100 and 220 nm. Virus growth in Xenopus cells is accompanied by the production of CPE and plaques, but growth in other cells (tick, Vero, pig kidney, BHK-21) is demonstrable only upon secondary assay. Growth in all of these cell lines was inhibited at incubation temperatures above 34°C, and could not be demonstrated in mouse fibroblast or mosquito cells. Although intracerebral (ic) inoculation of Cascade virus in suckling meadow voles caused sporadic illness and death, suckling Swiss white mice and hamsters, as well as wet chicks, showed no signs of infection after ic or peripheral inoculation, and the virus failed to grow in the yolk sac of embryonated hens' eggs. Although no evidence of human infection with Cascade virus was obtained from a small sample of sera selected from Californians who became sick after tick exposure, recent evidence in Oregon and California of tick-associated disorders of undetermined cause (erythema chronicum migrans, Lyme disease) suggests further study.

We will continue to study the applicability of arthropod- and other poikilothermic cell lines, especially the new line of *Rhipicephalus sanguineus* cells, to the study of vector-borne disease agents. These poikilothermic cells have proven extremely useful in the recognition of pathogens from man-biting ticks, which agents could not be isolated in conventional, homeothermic systems. They should also find use in the study of latency, diagnostic and prophylactic biologicals and control, both of vectors and of the pathogens they transmit.

Publications:

Yunker, C. E., Cory, J., and Meibos, H.: Continuous cell lines from embryonic tissues of ticks (Acari: Ixodidae). *In vitro* 17: 139-142, 1981.

Homsher, P. J. and Yunker, C. E.: Bilateral gynandromorphism in *Dermacentor andersoni* (Acarina: Ixodidae): morphologic and cytogenetic analysis. *J. Med. Entomol.* 18: 89-91, 1981.

Appendix 1. PL-480 Project 03-030-N. *In vitro* and *in vivo* Studies of Certain Tickborne Viruses in Vector and Host Cells, Al Azhar University, Cairo, Egypt

Annual Funding: $14,626

This project investigates the contrasting behavior of an African tickborne virus, Quaranfil (QRF), which is infectious for man in vector- as opposed to vertebrate-host tissues and the factors affecting virus transmission by specific tick species. Topics studied this year included QRF virus-induced disease in chickens, the effect of immunosuppression on QRF viremias, accumulation of QRF viral protein in vector salivary glands, and assay of QRF viral antigens in cultured cells by a variety of techniques, including a newly developed passive haemagglutination test. Five research articles were published this year. The agreement has been extended until April 1982, without additional funding, to permit completion of current projects.

Appendix 2. PL-480 Project 03-051-N. Biochemistry and Physiology of Tick Vectors of Disease.

Annual Funding: $52,952

This project provides basic knowledge concerning the chemical and physical environment in which tickborne pathogens exist when not infecting the vertebrate host. Areas investigated during the third year of the agreement included: conversion of tick-egg proteins to tissue proteins, the existence and characterization of isocitrate lyase in tick embryonic tissues (this mediator of the glycolytic pathway has not previously been reported for arthropods), purification and characterization of tick catalase and pyrophosphatase, and early embryonic development of the camel tick. This project will terminate December 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00200-02 EB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunobiology of Relapsing Fever

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. G. Stoenner	Asst. Sci. Director, NIAID (RML)
OTHER:	None	

COOPERATING UNITS (if any)

None

LAB/BRANCH

Office of the Scientific Director, NIAID

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is principally concerned with elucidating the pathogenesis of relapsing fever and the dynamics of seroconversion of the organism.

PHS-6040

Project Description:

Last year, major progress was made toward developing the reagents needed to investigate the dynamics of seroconversion of Borrelia hermsi. Twenty-four immunologically distinct serotypes were identified, infectious stock pools of each were made and fluoresceinated antiserums were prepared against each serotype. The primary objective of this project is to elucidate the mechanism by which seroconversion occurs. Many investigators contend that Borrelia constantly change their surface antigens, independent of any influence by the immune response of the host and that relapses represent new populations to which the host has not responded. Our findings to date, which are in conflict with this theory, suggest that seroconversion is initiated by a host response, probably a production of immune globulins.

We initially attempted to cause seroconversion by challenging the susceptibility of animals that had been infected 5 days before with a single serotype and then rendered free of Borrelia by antibiotic treatment. However, large challenges (75,000 organisms) were required before other serotypes emerged, and it was difficult to prove that the challenge dose was not contaminated with small numbers of these serotypes. Then, an attempt was made to cause seroconversion by mixing peritoneal exudate cells of immune animals with varying numbers (500 to 5,000/mouse) of Borrelia of the same serotype incubating the mixture for 2 to 3 hrs and then passing the mixture intraperitoneally to normal mice that served to amplify the population. Although some of the organisms in a small challenge dose converted, conversion was never complete because part of the challenge serotype always remained. Complete seroconversion was finally obtained by passive transfer of varying dilutions of convalescent serum to normal mice and then challenging them 4 to 5 hrs later with 1,000 Borrelia of the homologous serotype. This challenge dose was shown to be a pure population (up to 20,000/ml) of a single serotype. By this procedure, dominant serotypes were converted to minor ones, and vice versa, minor ones were changed to dominant serotypes. Typically seroconversions were induced by several 2-fold serum dilutions just greater than those that completely neutralize the homologous serotype. In future studies, attempts will be made to fractionate 4- to 5-day convalescent serum to identify the fraction(s) responsible for seroconversion. Further attempts will also be made to characterize the distinct antigens that separate each of the 24 serotypes from the rest.

Publications: None

Rocky Mountain Laboratory
Administrative and Operational Support
Operations Branch

1981 Annual Report

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Rocky Mountain Laboratories Operations Branch, NIH, NIAID

Administrative and Operational Support

October 1, 1980 - September 30, 1981

Introduction

The Branch provides all services necessary to the professional staff in the pursuit of their investigations. This support covers the following areas: procurement, personnel, custodial, security, media preparations, waste disposal, glassware refinishing, photography, animal rearing and care, motor pool, operation of power plants, and full maintenance in every area except electronics.

General Overview of the Responsibilities of Operations Branch

The fiscal and procurement department manages a budget of $1,107,000. Payroll is not included in this figure. It covers only the purchase of supplies and minor equipment used in the operation of the laboratories. Timekeeping and the correction of errors in the payroll are also handled in the unit.

Personnel handles all actions and advises on personnel matters. This department also is charged with the operation of the Comprehensive Employment Training Act (CETA) in association with the local Montana State Employment Office. Through the year we have averaged four (4) people on this program at all times. The maximum time a person may spend on the program is 3 months. Hence, we are constantly interviewing and employing people under the program. Also handled by Personnel are persons under the following programs: Stay-in-School, Work Study, Government Summer Program, Visiting Program, and students studying for advanced degrees.

Custodial services are provided in the five laboratory buildings daily. Security is provided in the form of a guard on duty every night of the year.

Most of the media used in the laboratories is prepared at the lab under a contract by an outside contractor. All glassware used in the lab is cleaned and sterilized for reuse in the laboratories.

The photography department provides full professional services necessary in the laboratories with the exception of medical artistry.

The animal unit raises one strain of guinea pig, eight strains of mice, one strain of rats, two strains of hamsters, and one colony of microtus. They breed and raise approximately 125,000 animals a year. An additional 10,000 animals are purchased annually from outside sources, including mink, sheep, rabbits, mice, and hamsters. After rearing, care is provided for these animals while they are under experiment.

The maintenance department, through the power plant, provides heat, steam, air and vacuum to the laboratories. Also provided are air conditioning, compressed air, and demineralized and distilled water. A motor pool consisting of 10 vehicles is maintained. Grounds care, including snow removal, is provided.

With the exception of the electronics work, all maintenance is done by the staff. This includes plumbing, electrical, sheet metal, carpentry, and refrigeration, including ultra low temperature boxes.

Labor management work is handled by the Chief of the Branch.

LABORATORY OF MOLECULAR MICROBIOLOGY
1981 Annual Report
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PHS-NIH
SUMMARY REPORT

ANNUAL REPORT OF THE LABORATORY OF MOLECULAR MICROBIOLOGY, NIAID
October 1, 1980 - September 30, 1981

Dr. Malcolm A. Martin
Chief, Laboratory of Molecular Microbiology

The Laboratory of Molecular Microbiology was established in May 1981 bringing together NIAID scientists using molecular approaches to investigate mechanisms of virulence and/or oncogenicity. Although many members of LMM have had a long-standing interest in animal virology and have concentrated their efforts on DNA and RNA tumor viruses, the basic thrust of research activities has been directed toward the examination of genome organization, regulation of gene expression, and the role of gene products in mediating different disease states. The DNA Recombinant Research Unit (DRRU) has employed molecular cloning techniques to study the organization and expression of DNA and RNA tumor virus genomes. During the past 4 years the DRRU has also been active in recombinant DNA activities at NIH and, since 1978, has conducted a variety of risk assessment experiments following consultation with investigators knowledgeable in the areas of bacterial virulence, plasmid biology, and intestinal physiology. It soon became apparent that expertise in these areas would be immensely beneficial to on-goining risk assessment activities and would fill a void in the intramural scientific program of NIAID. Consequently when the opportunity arose of hiring a well-trained plasmid biologist to work at the NIAID containment facility at FCRC and to use molecular approaches to study bacterial virulence, Dr. Donald LeBlanc was selected to initiate a program focussing on the molecular biology of Streptococcal plasmids and to advise NIAID staff in recombinant risk assessment matters.

During the past year the LMM scientific staff has carried out investigations in three program areas: 1) the oncogenic potential of papovaviruses; 2) the molecular structure of endogenous C-type retroviruses; 3) the molecular biology and genetics of streptococci. Considerable progress has been made in all three areas especially studies elucidating the organization of viral gene sequences integrated into the chromosomes of mammalian cells. The strength of LMM, which is in the areas of recombinant DNA technology, biochemical virology, and molecular genetics, has been complemented by the extremely productive collaborations with members of the Laboratory of Viral Diseases who have provided a wealth of expertise in the area of viral biology which has increased the breadth of our scientific programs.

An unexpected new development during 1980 was the discovery that normal human DNA contains sequences related to murine leukemia viruses (MuLV). Like the well characterized mouse system, human cellular DNA contains numerous (50 to 100) copies of endogenous C-type retroviral DNA sequences that resemble MuLV in size and organization. Molecular cloning techniques have been used to prepare more than 20 different clones from a human DNA library that are currently being characterized by restriction enzyme mapping, heteroduplex analysis, and nucleotide sequence determination. These clones of MuLV related human DNA represent potentially valuable reagents which can be used to monitor the etiology and course of human diseases. For example, the RNA present in leukemic white cells or in lymphomas can be evaluated for reactivity with defined subgenomic portions of cloned MuLV-related human DNA. These same DNA segments can also be inserted into appropriate animal viral vectors (such as SV40 or bovine papilloma virus DNAs) and introduced into mammalian cells where putative human analogues of gag, reverse transcriptase, and retroviral envelope genes may be expressed. Finally, it may be possible to synthesize novel oligopeptides whose amino acid sequence can be deduced from nucleotide sequencing studies. These synthetic peptide chains could then be used to raise antibody that would recognize putative MuLV related proteins in human cells.

The finding of human analogues of endogenous MuLV sequences provides a unique opportunity to apply a variety of state of the art techniques to answer fundamental questions involving human diseases. The power of recombinant DNA procedures, nucleotide sequencing, and hybridoma technology will be brought to bear to study the organization and expression of retroviral genomes in normal and malignant cells.

Use of an ecotropic MuLV env probe to identify endogenous proviruses present in mouse DNA. A 500 bp subgenomic segment which maps in the env region of AKR ecotropic proviral DNA was shown to specifically hybridize to ecotropic proviruses. When used in a series of blot-hybridization experiments of restricted mouse liver DNAs, results were obtained indicating that AKR/N, AKR/J and AKR/Boyse mice contained 3, 4 or 5, and 7 ecotropic proviruses. Similar experiments performed with "low-virus" ("low-leukemia") mouse DNAs indicated the presence of a single copy of proviral DNA in most instances (Khan, Martin; Rowe, LVD)

Construction and use of a xenotropic env specific DNA probe. A recombinant plasmid which contained a 500 bp DNA insert derived from the env region of a cloned NFS xenotropic provirus was used to assess the number and organization of related endogenous proviral DNAs in different inbred mouse strains. Preliminary results indicate the presence of multiple (15 to 20 copies) endogenous xenotropic proviruses which form a family of related, but distinct, proviral genomes. (Martin; Hoggan and Rowe, LVD)

Molecular cloning of MCF MuLV proviral DNAs. Two proviruses were cloned from AKR MCF 247 infected cells. One (MCF 1) consisted of a 6.8 kb segment that was colinear with the parental MCF 247 provirus and contained sequences extending from the 5' LTR to the env region. The second (MCF 2) was a deleted genome that proved to be an immensely useful reagent for the electron microscopic analysis of heteroduplex molecules. (Khan, Repaske, Martin; Rowe and Hartley, LVD; Garon, LBV)

Molecular cloning of endogenous MuLV proviruses from BALB/c and AKR mice. More than 40 clones were obtained from BALB/c and AKR mouse DNAs using lambdaphage vectors. The organization of viral genes and regulatory elements was determined and compared to comparable regions of known infectious MuLVs. (Khan and Martin)

Characterization of integrated polyoma virus genomes. Integrated polyoma genomes were molecularly cloned in lambdaphage vectors and characterized by restriction enzyme mapping, heteroduplex formation with polyoma viral DNA, and nucleotide sequence determination. Preliminary results suggests the existence of regions of polynucleotide sequence homology in flanking cellular and polyoma viral DNA sequences. (Chowdhury and Israel)

Preparation of monoclonal antibodies to polyoma virus tumor (T) antigens. Several hybridomas were established that specifically make antibody against different portions of polyoma large and middle T antigens. (Israel, Light, and Ito)

Protein kinase activity of polyoma virus middle T antigen. The protein kinase associated with polyoma virus middle T antigen was found to phosphorylate lipomodulin which, in turn, is a regulator of phospholipase A2. Since activation of the latter appears to be involved in stimulating mouse cells to enter DNA synthesis, the role of viral encoded middle T antigen in modulating cell division (and oncogenesis) is being carefully evaluated. (Ito)

Identification of tetracycline containing plasmids in Streptococci. Tetracycline resistance determinants were identified and partially characterized from *S. mutans* and *S. faecalis*. Conjugation experiments have also indicated that tetracycline and hemolysin plasmids of *S. faecalis* can form cointegrates containing drug resistance and hemolysin determinants on the same molecule. (LeBlanc)

Allograft-rejection resistance in SV40 transformed hamster cells and its possible role in tumor development. SV40 (a virus which produces tumors in hamsters) in contrast to adenovirus type 2 (a virus which does not produce tumors in hamsters) conveys a virus- and species-specific resistance to allograft rejection to Syrian hamster cells transformed *in vitro*. This finding implies that the induction of some type of resistance to the cellular immune defenses of the host may be essential to the process by which SV40 and other tumor viruses convert normal cells to tumor cells. (Lewis and Cook)

A spectrum of different tumor inducing capacities among adenovirus 2, adenovirus 12, and SV40 transformed hamster cells. Hamster cells transformed by these DNA viruses possess a spectrum of tumor-inducing capacities which vary from nononcogenic for immunoimmature newborns to highly oncogenic for immunocompetent syngeneic and even allogeneic adult hamsters. As there is no correlation between the immunogenicity of these transformed cells and their tumor-inducing capacity, these findings imply that DNA virus-transformed cells possess different resistances to rejection by the host's cellular immune system. Thus, DNA viruses seem to determine tumor-inducing capacities by conveying to the cells they transform different abilities to resist cellular immune rejection by the host. (Lewis and Cook)

Resistance to cellular immune rejection is an essential process in the conversion of normal hamster cells to tumor cells by oncogenic DNA viruses. DNA virus-transformed hamster cells which may or may not be oncogenic share many of the properties (such as immortality and the ability to grow in soft agar) with other transformed cells. Only transformed hamster cells which possess an inherent resistance to rejection by the hamster's cellular immune system are able to induce tumors in hamsters. Thus cell transformation (i.e., the conversion of a normal cell to a neoplastic cell with unlimited potential to divide) and the establishment of a pattern of resistance to cell-mediated immune rejection which results in oncogenicity for a specific host are independent events. Both of these events (i.e., transformation and conversion to a resistance phenotype) are required for tumor induction. (Lewis, Cook, and Hibbs)

New lymphotropic papovavirus. A new lymphotropic papovavirus isolated from African green monkey lymphocyte cultures has been further characterized biologically and biochemically. Serologic surveys of primate sera showed that a related virus is prevalent in virtually all primates including man. Because the original virus stock consisted of a high proportion of defective particles, the DNA of the virus was molecularly cloned using the pBR322 plasmid vector. The larger size cloned DNA (5100 bp) proved to be infectious and stocks of high titer, homogeneous, non-defective virus was thereby obtained. Tests of a number of other human lymphoblastic cell lines of T- B-cell origin using non-defective virus stocks indicated that the virus can replicate in both T- and B-cells, contrary to recent reports that this virus is strictly a B-lymphotropic virus. A preliminary physical map of the viral DNA has been constructed. (Takemoto and Yoshiike)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00190-03 LMM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Molecular Genetics of Eukaryotic Cells and Their Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Malcolm A. Martin	LMM, NIAID
OTHER:	Roy Repaske, Biochemist	LMM, NIAID
	Mark Israel, Medical Officer	LMM, NIAID
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SECTION
Biochemical Virology

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The principal arm of these investigations is the biochemical characterization of eukaryotic and prokaryotic genes and their associated regulatory elements. Attention has been focused on segments of papovaviruses and C-type retroviruses responsible for the establishment and maintenance of the transformed state. In this regard, the portions of polyoma virus DNA mediating tumorigenesis has been identified and integrated forms of the viral genome have been partially sequenced. The location and potential intracellular substrates of the protein kinase associated with polyoma virus middle T antigen have also been investigated. Subgenomic segments of MuLV proviruses have been used to identify endogenous retroviral sequences in mouse and primate DNA. DNA segments related to MuLV have been molecularly cloned from human DNA. Tetracycline resistance determinants have been shown to be components of R plasmids found in Streptococcus mutans and Streptococcus faecalis. Risk assessment experiments clearly indicate that potentially infectious viral DNA components of recombinant plasmids are not transferred out of EK1 derivatives of E. coli K12 following parenteral inoculation of susceptible animals.

PHS-6040
(Rev. 10-76)

Research Accomplishments

I. The Oncogenic Potential of Papovaviruses

A. Viral Genes Mediating Transformation (Israel and Chowdhury)

Previous studies from this laboratory have shown that polyoma virus (Py) large tumor (T) antigen (Ag) as well as those viral DNA sequences specifically encoding it could not be detected in some Py induced hamster and rat tumors. In contrast, rat cells transformed *in vitro* usually contain tandemly arranged complete copies of integrated Py DNA as well as Py large T Ag. Under a variety of *in vitro* growth conditions as well as following passage in animals (*in vivo*), Py transformed cells may lose tandem copies of the integrated viral genome as well as those sequences specifying large T Ag. Such transformed cells have undergone a change resembling tumor cells with respect to the organization of integrated viral DNA and the absence of large T Ag. To investigate whether the arrangement of viral DNA sequences in tumors represents the final stage of a multi-step process involving the elimination of tandemly integrated viral genomes and the rearrangement of Py DNA sequences following the primary integration event, several Py *in vitro* transformed rat cell lines, all of which contained tandemly integrated complete copies of viral DNA and synthesized large T Ag were passaged in animals. The organization of Py DNA sequences and viral encoded T Ags present in 14 tumor cell lines resulting from such a study were then evaluated. An unexpected finding was that functional large T Ag was detected in 13 of the 14 tumor cell lines. The stability of integrated viral DNA encoding large T Ag during the passage of transformed cells *in vivo* suggests that the distinctive organization of Py DNA in tumor cells is unlikely to have arisen by a multiple step recombinational process subsequent to the integration event associated with tumor induction.

Consequently, a number of recombinant phage clones containing integrated Py DNA and flanking host cell sequences derived from several virus induced tumors were carefully characterized. Despite what appears, at first glance, to be a colinear arrangement of integrated viral DNA sequences with the Py genome, detailed restriction mapping and nucleotide sequence determination has revealed the presence of deletions, substitutions, inversions, insertions, duplications and rearrangements of the integrated viral DNA sequences. Of particular interest, we believe, is our identification of stretches of patchy homology (7-20 bp) between viral DNA sequences and cellular DNA sequences flanking the integration site. These regions occur approximately 25 to 75 bp away from the viral-host cell junction and may serve to align the genomes during integration. Currently we are using cloned, flanking cellular sequences for the isolation of normal hamster embryo DNA segments corresponding to the site at which Py DNA integration occurs. Such clones should permit the characterization of the cellular integration site before its interaction with the viral DNA.

During the past year hybridomas producing monoclonal antibodies against the large and middle Py T antigens have been constructed and isolated. These hybridoma cell lines are being characterized for use in experiments to evaluate the functions of these important virus encoded proteins.

B. Viral Encoded Proteins and Transformation (Ito and Light)

It has been suggested that polyoma virus middle-sized T Ag (Py M-T) triggers oncogenesis via its associated protein kinase activity. Previous work from this laboratory has demonstrated that the phosphorylation of Py M-T occurs on the external side of the plasma membrane. During the past year this observation has been exploited in two ways: (1) A deletion mutant of polyoma virus, dl 1015 (Magnusson and Berg, J. Virol. (1979) 32, 523-529) has a Py M-T associated kinase activity when the middle T Ag of the mutant is solubilized from membrane preparations yet the mutant is incapable of transforming cells. The results of preliminary experiments suggest that the deletion present in dl 1015 may alter the conformation of the middle-T Ag in such a way so that its interaction with the plasma membrane is affected preventing the phosphorylation reaction from occurring on the cell surface.

(2) Lipomodulin is a cellular protein located on the external side of the plasma membrane which regulates phospholipase A_2 activity. Since the activation of phospholipase A_2 has been suggested as the rate limiting step in the series of events leading to the replication of 3T3 cellular DNA following the stimulation of quiescent cells by growth factors, it was of interest to assess the effect of polyoma virus (Py) infection or transformation induced by Py on the phosphorylation of lipomodulin. The results of such an experiment revealed a dramatic change in the lipomodulin phosphorylation pattern suggesting the possibility that Py M-T may alter the growth properties of cells by affecting, directly or indirectly, the phosphorylation of this membrane associated protein.

II. The Molecular Structure of C-Type Retroviruses

As was the case previously, these studies represent a collaborative effort between the Laboratory of Molecular Microbiology (LMM) and the Laboratory of Viral Diseases (LVD). The research activities fall into two major areas:

A. Organization of murine retroviruses

1. Construction and use of an ecotropic envelope (env) specific DNA probe to identify endogenous ecotropic MuLV proviruses (Moore and Rowe (LVD); Martin). An ecotropic env probe was hybridized to restriction enzyme cleaved liver DNAs prepared from 3 different AKR mouse colonies (AKR/N, AKR/J, and AKR/Boyse) and representatives of inbred "low-virus" and "low-leukemia" animals. The results of blot hybridization experiments indicated: a) all AKR mice contain the Akv-1 locus as well as a second provirus which has not been mapped genetically because it may not be expressed in the form of infectious virus; b) only AKR/N animals contain the Akv-2 locus which has been mapped to chromosome 16; c) AKR/N, AKR/J and AKR/Boyse animals harbor 3, 4 or 5, and 7 ecotropic MuLV proviruses; d) "low-virus" ("low-leukemia") mice such as BALB/c and C57 B6 usually contain a single copy of proviral DNA and, in rare instances, (strain SEA) may harbor a second provirus. Among this group of mice only 2 or 3 discrete loci were identified.

2. Construction of use of a xenotropic *env* specific MuLV probe (Hoggan and Rowe (LVD); Martin). A 500 bp DNA segment was subcloned from a recombinant lambdaphage containing a xenotropic provirus. This cloned *env* DNA segment annealed to xenotropic and MCF proviral DNAs and failed to hybridize to several ecotropic proviruses. The xenotropic *env* specific probe was then used to examine the number and organization of related endogenous proviruses in mouse cellular DNA. Results from hybridization experiments indicate: 1) that inbred mice contain at least 15 to 20 proviral DNAs that share envelope determinants with xenotropic MuLVs and 2) a family of related endogenous xentropic proviruses are present in all mice that differ from one another in the location of "multi-cut" restriction enzyme sites.

3. Molecular cloning of MCF MuLV proviral DNA [Khan, Repaske, Martin; Garon (LBV); Rowe (LVD)]. Two clones obtained by the shotgun cloning of MCF 247 MuLV infected mink cells in λWES were extensively characterized by restriction enzyme mapping, blot-hybridization, and nucleotide sequence determination. One clone (MCF-1) was colinear with authentic MCF 247 proviral DNA and contained envelope sequences that annealed to the xenotropic *env* specific probe. The second clone (MCF-2) contained a 1800 bp deletion in the *env* region and proved to be an invaluable reagent for electron microscopic heterduplex studies since it formed a characteristic deletion loop with DNA samples containing an *env* region and circular molecules with DNA segments containing a complete LTR. Nucleotide sequencing experiments showed that the LTRs of MCF 1 and MCF 2 proviral DNAs contained one and two copies, respectively, of the 104 bp directly repeating unit.

4. Restriction enzyme mapping of endogenous MuLV proviruses (Khan and Martin). Thirty three independent MuLV reactive clones obtained from a BALB/c library in λ Charon 4A and 9 other clones prepared by shotgun cloning of *Eco* RI digested AKR/J mouse DNA were purified and mapped with several restriction enzymes using subgenomic MuLV DNA a variety of probes. The endogenous proviral DNAs were then compared to the proviruses of known infectious MuLVs. Approximately 50% of the endogenous clones contained LTR sequences but in no case were the endogenous LTRs organized like analogues associated with infectious MuLVs. Although several proviruses were obtained that had envelope regions characteristic of xenotropic MuLVs, a few were isolated that possessed both ecotropic and xenotropic *env* determinants.

B. Organization of MuLV related sequences present in human DNA (Martin)

Employing low stringency hybridization techniques and a "boot-strap" cloning approach, an 11 kb DNA segment, which was cloned from African green monkey DNA because it annealed to a generalized MuLV probe, was used for the molecular cloning of related sequences from normal human DNA. A highly conserved cross-annealing retroviral DNA segment which encodes reverse transcriptase was found to be present in nearly all of the 20 human clones obtained. The MuLV related human DNA segments form a family of related sequences (similar to the endogenous xenotropic proviruses of the mouse) that differ from one another by the location of "multi-cut" restriction enzyme sites. Several of the human clones may contain LTR sequences and are being analyzed by heteroduplex and nucleotide sequencing techniques to establish their precise organization.

III. Molecular Biology and Genetics of the Streptococci (LeBlanc)

During the past year efforts have continued to determine the role of plasmids in streptococcal antibiotic resistance and carbohydrate metabolism. A number of tetracycline resistant streptococci, particularly strains of *S. mutans*, do not contain detectable plasmids nor is the resistance trait curable by standard procedures. However, resistance is often transmissible to other strains of the same species or to *S. faecalis*. Plasmid DNA was isolated from the latter species, and both resistance and plasmid were simultaneously cured. Such plasmids are currently being employed as probes to determine whether Tc resistance is on a similar plasmid or the chromosome of the original donor. One strain of *S. mutans*, exhibiting non-transmissible erythromycin (Em) resistance and possessing no detectable plasmids, was shown to contain DNA homologous to a plasmid-determined Em resistance determinant from *S. faecalis*.

A clinical isolate of *S. faecalis* was shown to possess two Tc resistance determinants, one on a transmissible R plasmid and the other on the chromosome. The chromosomal determinant, exhibiting no homology with the R plasmid, was transposable to a second plasmid in this strain which coded for β hemolysis. When the transposable determinant was integrated in one particular restriction fragment, the presence of low levels of tetracycline was required for the induction of hemolytic activity as well as tetracycline resistance. Conjugation experiments using this strain as a donor have revealed that the R plasmid and hemolysin plasmid can form different types of cointegrate molecules resulting in various combinations of resistance traits including resistance to Em, streptomycin, kanamycin and one or the other of the two Tc determinants, of the hemolysin plasmid following conjugation. These results have lead to experiments designed to establish a linkage map of the resistance genes on the R plasmid.

In a final group of experiments, the *S. sanguis* Challis transformation system was used to establish the presence of two structural genes for lactose metabolism on a lac plasmid from *S. lactis*. The two gene products, a lactose phosphoenolpyruvate-dependent phosphotransferase activity (lac-PTS) and a phospho-β-galactosidase activity (P-β-gal) replaced the corresponding chromosomally-determined activities in the appropriate Challis mutants following transformation. The plasmid-determined enzymes were readily distinguishable, biologically (lac-PTS) or physically (P-β-gal), from the corresponding Challis enzymes. Plasmid DNA could not be isolated from any of the lac$^+$ transformants under conditions which have yielded such elements from Challis isolates transformed by other plasmids.

During the next year we will examine in greater detail the nature of the transposable Tc resistance determinant from *S. faecalis*. These studies will include an examination of the effects of integration site and orientation on the regulation of hemolysis. Such information will be useful for identifying the physical location and studying the regulation of other plasmid-, as well as chromosomally-determined phenotypic properties among the streptococci. The use of transposons to produce polar mutations will also enhance our ability to determine whether operons exist in the streptococci.

The use of recombinant DNA technology will be required to answer questions concerning expression and regulation of several streptococcal properties. Preliminary results in other laboratories suggest that a number of streptococcal genes are not expressed, or their products not properly processed, by existing *E. coli* or *B. subtilis* host-vector systems. We have therefore begun using the Challis strain of *S. sanguis*, the pVA736 plasmid vector of Macrina, and the lac plasmid from *S. lactis*, containing structural genes for a cytoplasmic enzyme (P-β-gal) and a membrane-associated activity (lac-PTS), to establish a totally streptococcal recombinant DNA system. At the very least, the success of these experiments will result in the cloning of the two structural genes for use as probes to detect a plasmid or chromosomal location of these genes in the lac$^+$ Challis transformants described above.

IV. Assessment of Risks Associated with Recombinant DNA Research

(Drs. Martin and Rowe)

Previous risk assessment experiments have been extended to EK-1 derivatives of *E. coli* K12 containing a variety of viral (monomer and dimer) DNA inserts. In no case did animals inoculated with 10^7 to 10^8 live bacteria develop a viral infection (mice) or tumors (hamsters). One of 30 mice inoculated subcutaneously with 10^9 live *E. coli* containing a dilysogen of polyoma virus DNA had evidence of a virus infection.

Experiments are currently in progress to evaluate whether viral DNA inserts can be transferred out of wild type *E. coli* administered to conventional and germ-free animals.

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Chowdhury, K., Light, S., Garon, C.F., Ito, Y., and Israel, M.A.: A cloned polyoma DNA fragment representing the 5'-half of the early gene region is oncogenic. *J. Virol. 36*: 566-574, 1980.

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Heilman, C.A., Law, M.F., Israel, M.A. and Howley, P.M.: Molecular cloning of human papillomavirus genomes. *Journal of Virology 36*: 395-407, 1980.

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Smith, A.E., Fried, M., Ito, Y., Spurr, N., and Smith, R.: Is polyoma virus middle T antigen a protein kinase? *Cold Spring Harbor Symp. Quant. Biol. 44*: 141-147, 1980.

Honors

Dr. Martin continues to serve on the Editorial Board of the Journal of Biological Chemistry and regularly reviews papers for the Journal of Virology. During the past year he was invited to give seminars at Johns Hopkins University, Yale Medical School, Howard University, American Type Tissue Culture Collection, and two intramural laboratories of the National Cancer Institute. He is a member of the NIAID Risk Assessment Committee, the Executive Recombinant DNA Committee of NIH, and participated in the U.S. - Japan Cooperative Workshop held in Honolulu in February, 1980. During the year Dr. Martin carried out site visits for NCI and the Canadian National Cancer Society.

Dr. Israel was invited to be a principal speaker at an ASM symposium dealing with integrated viral genomes. He also gave lectures at the Uniformed Services School of Medicine and Walter Reed Army Medical Center and received an invitation to attend the Banbury Conference on Tumor Antigens at Cold Spring Harbor Laboratory, July 2-8, 1981.

Dr. Ito was invited to attend a workshop on "Tumor and Transplantation Antigens" at Cold Spring Harbor Laboratory, July 2-8, 1981.

Dr. LeBlanc was invited to present a paper dealing with Streptococcal plasmids at the International Plasmid Conference, January 5-9, 1981, Santo Domingo, Dominican Republic and delivered the annual John C. Hartnett lecture at St. Michael's College, Winooski, Vermont in October, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00018-15 LMM

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biological and Biochemical Characterization of Human Papovaviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Kenneth K. Takemoto, Head	LMM, NIAID
OTHER:	Kunito Yoshiike, Visiting Scientist	LMM, NIAID

COOPERATING UNITS (if any)

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Viral Biology

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3	3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During the past year, a major part of our efforts were devoted to the study of a newly discovered lymphotropic papovavirus (LPV) isolated from lymphocyte cultures of an African green monkey. This virus, belonging to the polyomavirus genus replicates exclusively in continuous lines of lymphoblastic cells. Serologic surveys indicate that similar viruses infect virtually all primates including humans. Because of the presence of defective particles in the original stock, the DNA of the virus was molecularly cloned using the plasmid vector pBR322. Analysis of 20 clones revealed the majority consisted of shorter length DNA molecules which were non-infectious. The longer DNA molecules were infectious and resulted in the production of high-titer, non-defective stocks of LPV. Using these new pools of LPV, we have established that the virus is not a strictly B-lymphotropic virus and can replicate in some lines of T-lymphocytes. A physical map of the LPV DNA has been constructed based on restriction endonuclease analysis of the cloned, non-defective DNA.

Project Description

I. Characterization of New Lymphotropic Papovavirus

A new and potentially important papovavirus was recently isolated from lymphocyte cultures established from the lymph nodes of an African green monkey. This virus, designated as lymphotropic papovavirus (LPV) is unusual in that it replicates only in dividing lymphocytic cells of monkey or human origin. Antigenic studies have shown that it is unrelated to any of the known members of the polyomavirus genus but shares the common group specific antigenic determinant of polyomaviruses. Our current studies have been directed toward a complete biological and biochemical characterization of the virus.

A. Serologic studies

Serologic evidence for related lymphotropic viruses in primates has been obtained after an extensive survey for antibody against LPV in various primate sera. Virtually all the non-human primates had antibody to LPV indicating the widespread occurrence of a similar, related lymphotropic papovavirus in these different animal species. Among normal humans, approximately 25% had antibody to LPV with fluorescent antibody titers of up to 1:40. This is strong evidence that humans are also infected with a lymphotropic papovavirus related to the African green monkey LPV.

B. Molecular cloning of LPV DNA

The original stock of LPV which was received from Dr. zur Hausen consisted mainly of defective particles, and viral replication was extremely slow. In order to obtain non-defective viral stocks and to study the viral DNA, the LPV DNA was molecularly cloned using pBR322 plasmid as the vector. Analysis of 20 cloned DNAs revealed that 1/4 were larger sized molecules (5100 bp) and 3/4 were of shorter length (4900 bp). Tests for infectivity showed that the larger size DNA was infectious while the shorter length molecules were defective. From infectious DNA, we have now obtained high titer, non-defective virus stocks which replicate faster and more efficiently in lymphoblastic cell lines.

Analysis of the molecularly cloned DNA with restriction enzymes have resulted in the construction of a preliminary physical map.

C. Host range studies

It has been reported that LPV replicates only in B-lymphocytes by zur Hausen, et al. A test of a number of T, B, and Null cells using high titer non-defective LPV has shown that this virus can replicate in at least one line of T-lymphoblasts derived from a patient with acute lymphoblastic leukemia. LPV is therefore not restricted to growth in B-lymphoblasts only.

D. Development of an assay for LPV

Up to the present time nothing was known concerning the infectivity of LPV due to the lack of an assay system for the virus. During the past year, a method for assaying the infectivity of LPV was developed based on the utilization of a variant of the B-lymphoblastic cell line, BJA-B. It was observed that although these cells generally grow as free-floating cells, some of the cells have the capacity to attach to the solid plastic substrate. These cells were selected by repeated removal of floating cells, and eventually resulted in the isolation of variant cells which could adhere to the plastic surface and form loose monolayers. When these cells, designated as M/BJA-B, were infected with LPV, infected cells tended to become detached from the surface. Using cell detachment as the indicator of infectivity, LPV stocks were assayed for infectivity and virus titers of 10^7 or greater has been observed in various stocks.

II. Analysis of DNA of JCV adapted to growth in human embryonic kidney cells

Last year, we reported on the adaptation of JCV to growth in human embryonic kidney cells. This was necessary because of the narrow, restricted host range of JCV which up to the present time had been grown only in human fetal brain cells. The DNA of the adapted JCV was analyzed after being molecularly cloned and found to consist of 2 kinds of defective molecules. One type had a major deletion in the early region and the other a major deletion in the late region. The 2 types of particles were thus shown to replicate by complementation.

In order to gain further understanding of the mode of replication of the HEK-adapted JCV, the DNA obtained after cloning in pBR322 is currently being sequenced.

Publications

Takemoto, K.K.: Human polyomaviruses: evaluation of their possible involvement in human cancer. Cold Spring Harbor Symposium on Viruses in Naturally Occurring Cancers. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York. 7: 311-318, 1980.

Israel, M.A., Martin, M.A., Miyamura, T., Takemoto, K.K., Rifkin, D., and Pollack, R.: Phenotype of polyoma-induced hamster tumor cell lines. J. Virol. 35: 252-255, 1980.

Miyamura, T., Yoshiike, K., and Takemoto, K.K.: Characterization of JC papovavirus adapted to growth in human embryonic kidney cells. J. Virol. 35: 498-504, 1980.

Howley, P.M., Rentier-Delrue, F., Heilman, C.A., Law, M-F., Chowdhury, K., Israel, M.A., and Takemoto, K.K.: Cloned human polyomavirus JC DNA can transform human amnion cells. J. Virol. 35: 878-882, 1980.

Honors

Awarded fellowship from Japan Society for Promotion of Science and appointed as Visiting Professor, University of Tokyo, Tokyo, Japan.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-0013-18 LMM

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TITLE OF PROJECT (80 characters or less)

Studies of Viral Antigens in Virus-induced Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Andrew M. Lewis, Jr. Research Virologist, LMM, NIAID

COOPERATING UNITS (if any) Arthur Levine, Cephas Patch, Alan Rabson (NCI); Heiner Westphal, (NICHHD) James L. Cook, National Jewish Hospital, John B. Hibbs, University of Utah Medical Center.

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The initial objectives of this project were to use human adenoviruses and adenovirus-SV40 (Ad2-SV40) recombinants as tools to study the genetics of DNA tumor viruses, to define the role of viral genes and viral antigens in viral oncogenesis and to study the biology of Ad2-SV40 recombinants. Due to the lack of proper containment facilities in NIAID between January 1973 and May 1978, there was a 5-year disruption (see past reports) in the major thrust of this project. During this interval, a study of Ad2-SV40 recombinants associated tumor induction in Syrian hamsters by these agents with the incorporation into the adenovirus-2 chromosome of a specific segment of SV40 DNA. To begin to understand the mechanisms by which this SV40 DNA segment conveys oncogenicity to these recombinants, it was necessary to understand why Ad2 was nononcogenic and why SV40 was highly oncogenic for hamsters. Since Ad2 and SV40 can transform hamster cells in tissue culture, we initiated a study of the oncogenic properties of cells transformed by these viruses. The results thus far lead us to believe that SV40 conveys to transformed hamster cells the ability to resist rejection by the host's cellular immune system.

Conversely, adenovirus 2 appears to be unable to convey resistance to cellular immune rejection to the cells it transforms. These data imply that, in addition to transformation, the induction of some type of resistance to cellular immune rejection is essential to the process by which SV40 converts normal cells to tumor cells. The incorporation of the segment of the SV40 genome which induces this resistance to cellular immune rejection into the adenovirus 2 chromosome could explain the mechanism by which SV40 conveys oncogenicity to certain adenovirus 2-SV40 recombinants.

Major Findings

The discovery of allograft-rejection resistance in SV40 transformed hamster cells - its possible role in tumor development.

SV40 is an oncogenic DNA virus that induces tumors *in vivo* when innoculated into rodents and transforms cells *in vitro* from a variety of species, including rodents, primates, and humans. Tumor induction by SV40 appears to be unique to Syrian hamsters, mastomys, and certain strains of mice because no virus-induced tumors have been observed in rats, guinea pigs, rabbits, monkeys, and humans inoculated with this virus. With the exception of cells from Syrian hamsters, cells transformed *in vitro* by SV40 usually do not produce tumors when inoculated into autologous or isologous hosts; however, such nononcogenic, SV40-transformed cells will frequently grow as tumors in immunologically compromised animals. Thus, the oncogenicity of SV40 is species specific, and the lack of oncogenicity of SV40-transformed cells for an appropriate host appears to be due to the inability of these cells to escape the immunological responses of the recipient. Available data imply that the early SV40 proteins (encoded prior to viral DNA synthesis) are responsible for the transformed state that, in turn, results in tumor development in hamsters. However, the lack of oncogenicity of SV40 for most other species and the lack of oncogenicity of SV40-transformed cells containing the early SV40 proteins suggest that species-specific factors restricted to hamsters are also involved in SV40's tumor-inducing capacity. To explain the susceptibility of hamsters to SV40 tumor induction, it has been generally assumed that these animals are relatively immunoincompetent compared to other mammals. However, recent data suggest that the immunological responsiveness of hamsters is not significantly different from that of other species. These data include the expression of strong cellular and humoral alloimmune reactions and the thymus-dependent rejection of Ad2-transformed cells. Another explanation for the species-specific oncogenicity of SV40 and SV40-transformed cells is that SV40 might induce a unique change during transformation of hamster cells that renders the resulting neoplastic cells resistant to host rejection. By studying the efficiency of tumor induction by SV40 and Ad2-transformed inbred hamster cells and transformed cell-induced tumors, we have developed data that supports this possibility. Four clonally-derived SV40 transformed inbred hamster (LSH strain) embryo cell lines induced tumors with equal efficiency in both syngeneic adult LSH and allogenic (as shown by skin graft rejection, mixed lymphocyte reaction, and graft versus host reaction) CB hamsters. The average number of SV40 transformed cells per tumor producing dose at the 50% end-point (TPD$_{50}$) for LSH hamsters was $10^{4.0}$ while the average TPD$_{50}$ for CB hamsters was $10^{4.9}$--a difference that was not statistically significant.

Three Ad2 transformed LSH embryo cell lines which induced tumors in newborn hamsters, failed to induce tumors in either syngeneic LSH or allogeneic CB adults (TPD$_{50}$'s $\geq 10^{8.3}$). Seven SV40 transformed cell-induced tumors also produced tumors with equal efficiency in both syngeneic and allogeneic hamsters; the TPD$_{50}$ LSH/TPD$_{50}$CB varying only 5 fold. In contrast, 4 syngeneic adult adapted (by serial passage in newborns) Ad2 transformed cell-induced tumors produced tumors efficiently only in syngeneic LSH adults; the TPD$_{50}$LSH/TPD$_{50}$CB varying >1,000 fold.

These results shown that SV40, in contrast to Ad2, conveys what appears to be a species-specific resistance to allograft rejection to transformed Syrian hamster cells and imply that, in addition to transformation, the induction of some type of resistance to rejection by the cell-mediated responses of the host is necessary to the process by which SV40 (and possibly other tumor viruses) convert normal cells to tumor cells.

The resistance of SV40 transformed hamster cells to the cytolytic effect of activated macrophages as a possible factor in tumor induction.

To begin to evaluate the reasons for the apparent resistance of SV40 transformed hamster cells to host cell-mediated defenses, (Cook, Hibbs, Lewis), we have studied the effects of "activated" macrophages from animals with chronic intracellular infections on SV40- and Ad2-transformed rodent cells. The battery of target cells evaluated displayed three different transformed cell phenotypes when inoculated into animals--nononcogenic, oncogenic in the host of origin but not histoincompatible host, and oncogenic in both the host of origin but not histoincompatible host, and oncogenic in both the host of origin and histoincompatible host--and provided the opportunity to study the relative susceptibilities of these different target cell types to killing by activated macrophages. Activated macrophages are one of the effector cell components of cellular immune reactions that have been shown to inhibit the growth of or to lyse neoplastic cells *in vitro*. The tumoricidal effect of these activated macrophages is mediated by a nonphagocytic mechanism that requires intimate contact with target cells. The results obtained thus far suggests that resistance to the cytolytic effect of tumoricidal, activated macrophages is one of the mechanisms by which SV40 transformed hamster cells may evade destruction by the host.

Immunogenicity of Ad2 transformed hamster cells and transformed cell-induced tumor lines.

To determine the possible role of Ad2 transplantation antigen (TSTA) in the rejection of tumors transplanted to weanling hamsters, several types of studies are underway. Assays have been developed for Ad2 TSTA using an Ad2 tumor line (Ad2HTL1), which had been adapted to grow in adult hamsters or cells from adult adapted tumors grown in tissue culture (Ad2HTL3-1). Immunization with Ad2 significantly protected (defined as a resistance index *RI) greater than 10) hamsters against a challenge with either of these tumor cells. Immunization with SV40 did not convey significant protection (RI between 1 to 10) in these experiments. This assay is being used to study the immunogenicity of Ad2 transformed cell lines and newborn and weanling tumor lines developed from these transformed cells. Results thus far indicate that 5 Ad2 transformed cell lines (Ad2HE1, Ad2HE3, Ad2HE6, Ad2HE7, Ad2HE9) and the tumor lines established from three of them (Ad2HTL1, Ad-2HTL3, Ad2HTL6 contain a common Ad2 TSTA which during immunization with x-irradiated cells conveys protection to hamsters challenged with either of the Ad2 tumor cells. Differences in RI did not correlate with the differences in the transplantability of the newborn tumor lines to newborn and weanling hamsters. Adult hamsters that had rejected tumors induced by viable Ad2HTL3 and Ad2HTL6 cells were also protected when challenged with Ad2HTL1. These results show that whether they are accepted or rejected by weanling hamsters, each of these lines contains detectable Ad2 TSTA. Such findings appear to exclude the possibility that certain tumor lines can be transplanted to older animals because they lose detectable Ad2 TSTA. To determine whether a reduction in the concentration of Ad2 TSTA is responsible for the transplantability of certain newborn tumor lines to adults, the amount of immunizing antigen in suspensions of different tumors will be evaluated.

A Spectrum of tumorigenic phenotype among adenovirus 2, adenovirus 12 and SV40 transformed Syrian hamster cells.

We (Lewis, Cook) have developed adenovirus 2, adenovirus 12 and SV40 transformed cell lines which represent a spectrum of tumor-inducing capacities. This spectrum defines 4 distinct tumorigenic phenotypes: Type I - nononcogenic for newborn hamsters but oncogenic for nude mice; Type II - oncogenic for newborn hamsters but nononcogenic in syngeneic adults: Type III - oncogenic for both newborns and syngeneic adults; Type IV - equally oncogenic for syngeneic and allogeneic adult hamsters. Modulation of the hamster's cellular immune system by immunosuppression or exposure to histoincompatible cells in second set allograft reactions alters the tumor-inducing capacity of transformed cells expressing 3 of the tumorigenic phenotypes. A strong correlation exists between the ability of transformed hamster embryo cells expressing phenotype IV to produce tumors in allogeneic CB hamsters and their resistance to cytolysis *in vitro* by activated macrophage. As there is no correlation between the immunogenicity (determined by the induction of virus-specific transplantation immunity) of 4 transformed cell lines and their tumor-inducing capacity, the data suggest that DNA virus-transformed cells possess different resistances to rejection by the hosts' cellular immune system. Thus, DNA viruses seem to determine the tumorigenic phenotype by conveying to the cells they transform different abilities to resist cellular immune rejection by the host.

Resistance to cellular immune rejection as an essential process in the conversion of normal hamster cells to tumor cells by oncogenic DNA viruses.

The concept that viruses convert normal cells to tumor cells by transformation is one of the basic precepts of viral carcinogenesis. Observations that cells from certain species transformed by some DNA viruses are highly tumorigenic for immunocompetent hosts support this concept. However, cells from the same species transformed by different viruses can be so weakly oncogenic that immunoincompetent hosts must be used to demonstrate any tumor inducing capacity. Furthermore, viruses that produce highly oncogenic transformed cells in one species can produce transformed cells from the same or other species that are very weakly oncogenic. Other than suspected differences in the expression of viral-specific cell surface transplantation antigens, there are no satisfactory explanations for the differences in tumor inducing capacities expressed by DNA virus transformed cells. By characterizing a number of Ad2, Ad12, SV40 and polyoma virus transformed inbred hamster cell lines, we (Lewis, Cook, Hibbs) have defined a spectrum of tumorigenic phenotypes based on the efficiency with which the cell lines induce tumors in different types of hosts. The data suggest that, in addition to their immunogenicity, transformed cells must possess an inherent resistance to rejection by the host cellular immune response before they can be oncogenic in the species of origin. Since all of the types of cell lines to be described share many of the generally accepted *in vitro* properties of transformed cells but can be segregated into distinctly different tumorigenic phenotypes, we conclude that cell transformation (i.e., the conversion of a normal cell to a neoplastic cell with unlimited potential to divide) and the establishment of a pattern of resistance to cell-mediated immune rejection which results in oncogenicity for a particular host are independent events.

Correlation of resistance to cellular-immune, effector-cell killing in vitro with the tumor inducing capacity of DNA tumor virus transformed hamster cells.

We (Cook, Hibbs, Lewis) have recently found that SV40 and polyoma transformed hamster cells (Tumorigenic phenotype IV) which are highly oncogenic for both syngeneic and allogeneic adult hamsters ($10^{2.5}$ to $10^{4.5}$ cells/TPD50) are resistant to killing in vitro by activated macrophages (% specific ^{3}H thymidine released 16 to 22%). Adenovirus 2 and adenovirus 12 tranformed hamster cells (tumorigenic phenotypes II and III)which are almost nononcongenic for allogeneic adult hamsters (10^6 to > 10^8 cells/TPD50) are sensitive to killing in vitro by activated microphages (% specific ^{3}H thymidine released 70-96%). These findings suggest that resistance of transformed cells to the cumulative activity of effector cell populations that have obtained functional maturity in the host at the time of challenge may be one of the mechanisms that determines the cells' tumor-inducing capacity.

Selection of viral progeny with specific DNA modifications involving the loss of RNA splice signals during the replication of the nondefective hybrid Ad2+ND_4.

The molecular mechanisms by which animal viruses adapt themselves to replicate in cells from different species and in different cells from the same species are poorly understood. The inherent defenses within a prospective host cell against possible viral intruders are pierced only by those viruses which have mastered the task of altering their gene control mechanisms to meet the requirements of the new host. To expand our understanding of virus replication and virus-host cell interactions, it would be useful to develop model systems to study the ways in which viruses become capable of growing in different hosts. We (Gerry, Martin, Lewis, Westphal) have found that the nondefective recombinant between adenovirus 2 and SV40, Ad2+ND_4, adapts in an unique way to optimal growth in different host cells. In some lots of monkey cells, viable progeny (capable of independent growth) seem to retain splice signals in their DNA that are recognized by that host. In other lots of monkey and in human cells, viable progeny have lost the DNA region containing these splice signals. The affected DNA sequences are part of the early region of SV40 which is incorporated into the adenovirus 2 chromosomes during the formation of the Ad2+ND_4 recombinant. At this time, we speculate that the adaption of this virus to replicate in different host cells may be determined at the level of mRNA maturation. Studies are underway to further define this concept.

Significance to Biomedical Research

Our ability to expand our understanding of infectious agents and the diseases they produce requires the continued development of laboratory models which are amenable to study. The foregoing report documents the progress we have made in developing 2 new model systems which, hopefully, will provide fundamental insights into 2 important aspects of viral infections, namely, the ability to produce tumors and the capacity of viruses to parasitize different host cells.

Although many reports have documented the inability of virus-transformed cells to induce tumors in appropriate hosts and the lack of association between cell transformation in tissue culture and tumor induction in animals by oncogenic viruses, there has been no satisfactory explanation for this discrepant behavior. To understand these phenomena, model systems offering possible explanations must be developed and studied. We are optimistic that the Ad2/SV40 transformed hamster-cell system we are studying will be useful in beginning to provide a definitive explanation of the essential factors associated with the conversion of normal cells to tumor cells by oncogenic viruses.

The recently described phenomenon of RNA processing introduces a new level of regulation of molecular events in eukaryotic cells. The influence this regulatory system has on animal virus replication are currently being defined. Our studies thus far suggest that the virus-host cell systems associated with the replication of the nondefective Ad2-SV40 hybrid, Ad2+ND_4, will be useful in studying the effect of RNA processing on the ability of this recombinant to replicate in different host cells. Information from such studies could be important in assessing and controlling the attenuation of viruses for the development of live virus vaccines.

Publications

Cook, J. L., Hibbs, J. B., Jr. and Lewis, A. M., Jr.; Resistance of Simian virus 40 - transformed hamster cells to the cytolytic effect of activated macrophages - a possible factor in species-specific oncogenicity. Pro. Nat. Acad. Sci. 77: 6773-6777, 1980.

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